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CONTENTS

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SEC. D.—ZOOLOGICAL SCIENCES

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VOL. 17, SEC. C. FEBRUARY, 1939 NUMBER 2

AN ATTEMPT TO HYBRIDIZE ANNUAL AND PERENNIAL AVENA SPECIES[1]

BY L. P. V. JOHNSON[2] AND H. A. MCLENNAN[3]

Abstract

Cross pollination of annual with perennial Avena species did not result in the production of hybrid seeds, but certain specific combinations produced ovary stimulation.

Success in hybridizing annual Triticum and perennial Agropyron species (1, 2) and the resulting interest in the production of new, perennial forage crops prompted attempts to hybridize annual and perennial Avena species. The objective was the production of a perennial plant having the general foliage and seed characters of annual, cultivated oats. However, after two years' work the results, herein reported, are essentially negative.

Nine annual and eight perennial Avena species were used (see Table I). The annual species *A. sativa* and *A. byzantina* were represented by six and two varieties, respectively, while the perennial species *A. pratensis*, *A. planiculmis* and *A. pubescens* were represented by six, four and two forms, respectively.

Using the annual species as maternal material, a total of 2,914 florets were emasculated and cross-pollinated (1,652 in 1937 and 1,262 in 1938). A total of 56 crossing combinations, not distinguishing between strains, were attempted. In most cases the 1938 work repeated the combinations attempted in the previous year.

The annual (and biennial) species were started in the greenhouse in late winter and transplanted to the field in the spring in order that flowering might coincide with that of the perennial species (early mid-June). In 1937 about 300 florets on potted plants were emasculated and cross-pollinated in the greenhouse. The hybridization technique was to emasculate the florets of the annual plants about three days prior to anthesis, bag with glassine envelope, and pollinate with perennial species three days later. Further details on the general technique used are outlined in a previous report (3).

Table I presents data on the material used and the number of florets cross-pollinated in each crossing combination attempted. No hybrid seeds were

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Contribution from the Division of Forage Plants, Central Experimental Farm, Ottawa, Ontario. Published as N.R.C. No. 791.
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obtained from any of the cross pollinations made. In the case of *A. sativa* var. Early Ripe pollinated by *A. pratensis* 1422 and by *A. montana* 1430, a number of definitely stimulated ovaries were observed (Fig. 1). It is questionable whether this indicates closer phylogenetic affinities between these forms than between forms which produced no ovary stimulation upon cross pollination. Such stimulation is not considered to be the result of actual fertilization, but rather the result of activation by a chemical agent produced by the foreign pollen, probably in the course of germination.

TABLE I

THE NUMBER OF FLORETS OF ANNUAL SPECIES CROSS-POLLINATED BY PERENNIAL SPECIES

Female parent (annual Avena spp.)	Male parent (perennial Avena spp.)									
	A. elatior L. 113[1]	*A. versicula* Vill. 1420	*A. pubescens* L. 1421, 1435	*A. pratensis* L. 1422, 1426, 1427, 1429, 1433	*A. pratensis* L. var. Hertifolium, 1432	*A. planiculmis* Schrad. 1423, 1425, 1431, 1436	*A. montana* Vill. 1430	*A. bluvii* Asch. 1434	*A. compressa* Tleuff 1437	*Totals*
A. sativa L. var. Victory				34		303			76	413
A. sativa L. var. Gopher				61	83	48	29	15		236
A. sativa L. var. Selection 76	24	18	12	105	38	57	30	29		313
A. sativa L. var. Eagle						58				58
A. sativa L. var. Black Bountiful[2]						21				21
A. sativa L. var. Sixty Day	10	69	51	23	85	60	35	6		339
A. byzantina C. Koch						16				16
A. byzantina var. Early Ripe	12	23	66	80[3]	64	200	56[3]	11		512
A. nuda L.			8	26	7	124	23	29		217
A. fatua L.				6		29				35
A. sterilis L.				10		162				172
A. abyssinica Hochst.				9		87			33	129
A. strigosa Schreb.				42		101				143
A. wiestii Steudel		21		18	11	202		24		276
A. brevis Roth.				34						34
Totals	46	131	137	448	288	1468	173	114	109	2914

[1] *Numbers refer to strains within species or varieties.*
[2] *Winter variety (biennial).*
[3] *Definite stimulation of certain cross-pollinated ovaries.*

It is concluded that the annual and perennial Avena species in question are either not intercrossable or are intercrossable to a degree which is insufficient to produce positive results within the limits of the present work. Further attempts to intercross annual and perennial Avena species should, accordingly, be more extensive than the present investigation both in the number of combinations and in the number of cross-pollinated florets in each combination.

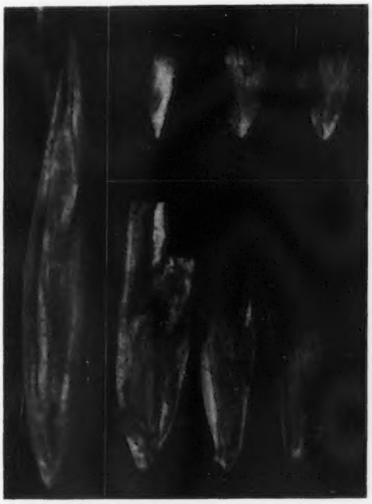

FIG. 1. *Left, normal seed. Upper right, unstimulated ovaries. Lower right, stimulated ovaries.*

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STUDIES ON *POLYMYXA GRAMINIS*, N. GEN. N. SP., A PLASMODIOPHORACEOUS ROOT PARASITE OF WHEAT[1]

By G. A. Ledingham[2]

Abstract

A new member of the Plasmodiophorales with rather unusual characteristics has been found parasitizing the roots of wheat grown in soil from three different localities in Ontario. In addition to spore clusters of the *Ligniera* type, large, septate zoosporangia with conspicuous tubes for zoospore discharge are present. These multinucleate zoosporangia are produced by progressive lobular outgrowths from uninucleate amoebae and from the beginning are always surrounded by a thin wall. In the formation of resting spores, naked multinucleate myxamoebae develop first, then segment to form spore clusters without formation of a soral membrane. Both zoosporangia and resting spores produce identical zoospores with two flagella of unequal length.

The somatic nuclear divisions in the growing myxamoebae are characterized by the simultaneous division of both the nucleolus and chromatin within a persistent nuclear membrane. During the transitional phase which follows, the nucleolus disappears and at the same time there is an intensification of the staining properties of the surrounding cytoplasm. Prior to segmentation of the myxamoebae to form the spore clusters, and in all divisions during growth of the zoosporangia, the nucleoli and nuclear membranes disappear, and divisions are of ordinary mitotic type.

Relationship with the Plasmodiophorales is indicated by the form of the resting spore clusters, the method of nuclear division during growth of the myxamoebae, and the characteristic flagellation of the zoospore. Since the zoosporangial characteristics of this fungus differ from those of other genera within this order, it is considered desirable to place it in a new genus for which the binomial *Polymyxa graminis* is proposed.

Introduction

Apart from *Plasmodiophora brassicae* and *Spongospora subterranea*, which have been studied quite intensively on account of their world-wide economic importance, few species of the Plasmodiophorales have received much attention from plant pathologists. It was of interest therefore to find a member of this order present as an intracellular parasite in the roots of wheat, as no previous record of parasitism of cereals by the Plasmodiophorales could be found.

The fungus was first discovered in the autumn of 1929 during an investigation of certain root rots of wheat occurring in Ontario. During the following three years, studies were made on the organism in the Botany Department, University of Toronto, whenever infected roots were available. Later, special studies on germination of the resting spores and morphology of the zoospores

[1] *Manuscript received January 26, 1939.*

This paper constitutes part of a thesis submitted to the Graduate School of the University of Toronto in partial fulfilment of the requirements for the degree of Doctor of Philosophy. The project was started at the University of Toronto in 1929 and carried on there during the tenure of two National Research Council Scholarships, and a research assistantship from the Department of Botany. Further studies were made at the Horticultural Experimental Station at Vineland, Ontario, and in the Laboratories of Cryptogamic Botany at Harvard University in 1932. The work has been completed in the Division of Biology and Agriculture of the National Research Laboratories. Published as N.R.C. No. 792.

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were made in the Laboratories of Cryptogamic Botany at Harvard University (7). In recent years most of the earlier observations have been rechecked and additional cytological studies have been made in the National Research Laboratories, Ottawa.

Resting spores were discovered first but it was soon found that large chytrid-like zoosporangia were associated with them in the wheat roots. It was thus of primary importance to determine whether these zoosporangia and resting spores belonged in the life cycle of the same organism. As a result of cultural and morphological studies there is now no doubt that this is so. The following reasons are given for this conclusion:

(1) Spore clusters and zoosporangia both developed in roots of wheat seedlings growing in nutrient solutions when inoculations were made with zoospores from zoosporangia only.

(2) Wheat plants grown in sterilized soil which had been inoculated with three-year-old, dried, finely powdered, infested roots produced both stages of the organism. Since it is very unlikely that the thin-walled zoosporangia can survive after drying, both types of thalli probably developed from germinating resting spores.

(3) The organism has been under observation for nine years and the two stages have invariably been associated on wheat from many different plantings in soil from different localities.

(4) During development, zoosporangia are most abundant at first, whereas the resting spores appear in greater numbers as the roots become more heavily infected. This would not be expected if the zoosporangia and resting spores were different organisms.

(5) Both spore clusters and zoosporangia produce identical zoospores of a characteristic biflagellate type which have been found to occur among the lower fungi only in the Plasmodiophorales.

As a result of the studies that have been made on this organism to date, the life history has been fairly well established. Such points as remain to be investigated are of a type on which information is still lacking on even the oldest members of the order. It seems advisable therefore to present the work as it now stands, leaving these questions for future consideration.

The absence in North American herbaria of all but a few species of the Plasmodiophorales, and the fragmentary accounts in the literature of some of the life histories, made it extremely difficult to determine the affinities of this new species. However, during a visit to Great Britain, the writer recently took advantage of the opportunity to compare it with most of the known species in all the genera of the order and was thus able to settle definitely the problem of relationship and nomenclature.

For reasons that will be discussed later it appears best to establish a new genus and species. Hereafter the binomial *Polymyxa graminis* will be used to designate this plasmodiophoraceous root parasite of wheat.

Material, Methods, and Host Relationships

The fungus was discovered in Marquis wheat roots that had been grown in soil obtained from the Central Experimental Farm at Ottawa. Later, wheat plants growing in the strawberry beds of the Vineland Horticultural Station were found to be infected, and soil from the Agricultural College Farm at Guelph also contained the organism. The heaviest infections of wheat were obtained in the greenhouse at about 60 or 65° F., using soil from Ottawa that had been frozen or dried for a few months.

Since there were no obvious external symptoms of disease in the host, such as hypertrophy or discoloration of the roots, microscopic observation was the only available method for detecting the presence of the fungus. Water mounts of living roots were quite satisfactory after thorough washing and removal of the air from the tracheids. However, the fungus stained so clearly with acid fuchsin or cotton blue in lacto-phenol that it was usually preferable to study stained material. If the roots were fixed in Bouin's or chrom-acetic fixer before lacto-phenol was added even nuclei were well preserved, but for fine cytological details microtome sections were superior. Difficulties due to hardening of the xylem in xylol were avoided by using an n-butyl alcohol series during the dehydration process.

Studies were made mainly with Marquis wheat as the host plant, but during the course of the investigation it was observed that Kubanka and Dawson's Golden Chaff wheat are also susceptible. In addition barley and rye, but not oats, have been infected. A similar type of resting spore was found in the roots of species of *Agropyron*, *Scolochloa*, *Rumex*, and *Impatiens*, but zoosporangia were not always associated with these. Several species of *Juncus* and *Poa* on which *Ligniera* had been reported in Europe did not become infected when grown along with parasitized wheat roots.

Morphology and Life History Studies

The Zoosporangia

The zoosporangium begins its development in the cortical cells of the wheat root as a uninucleate spherical thallus about three to four μ in diameter. The presence of a delicate surrounding membrane distinguishes it from the naked amoeba of similar size that will ultimately produce the resting spores. Nuclear divisions soon produce a multinucleate thallus, which may either remain spherical or become greatly elongated (Plate II, Fig. 14). The next step in the process is the production of a blunt lobular process from one side of this primordial thallus. A single nucleus moves out into this lobe and becomes much enlarged. Divisions follow and the new lobe becomes multinucleate and in turn sends out other lobes. In this manner a large septate thallus is developed which is completely surrounded from the beginning by a thin membrane (Plate I, Figs. 6, 7, 9). Although most of the lobate outgrowths are round or barrel-like in appearance, they may be very long and tubular, extending almost the entire length of the host cell. Development may then cease or a series of short blunt lobes may form at the end (Plate I,

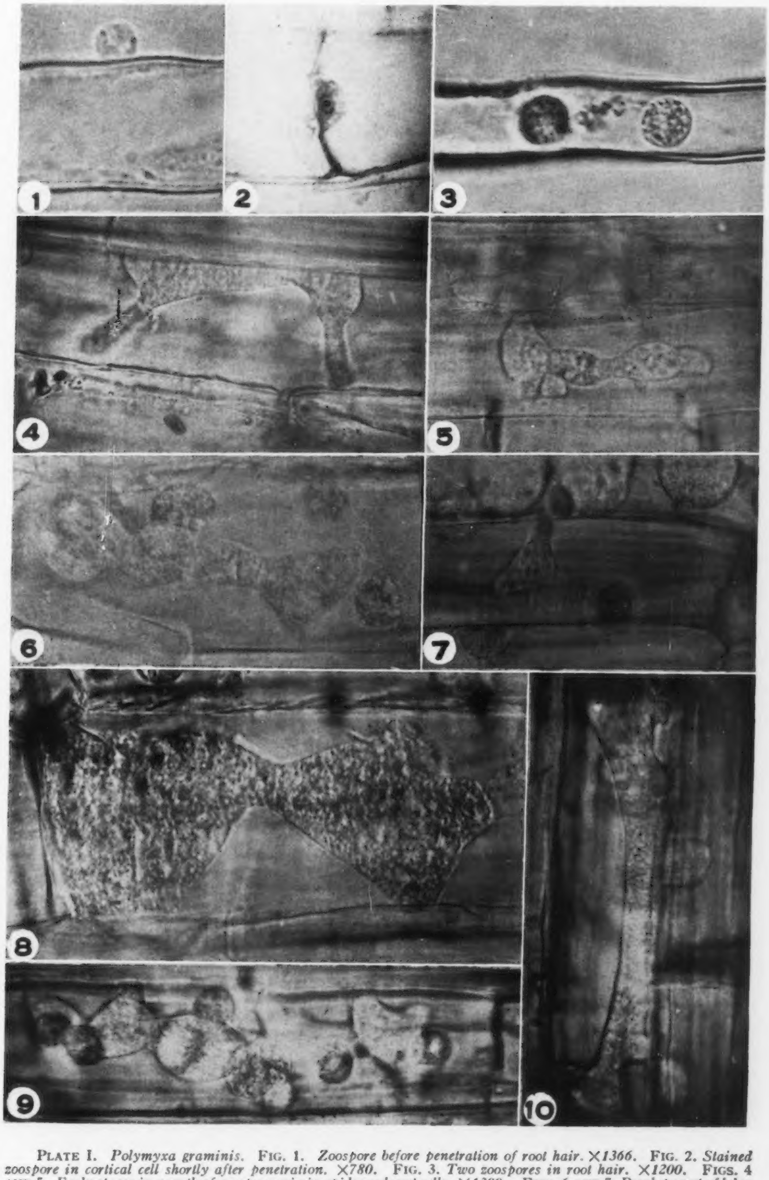

PLATE I. *Polymyxa graminis.* FIG. 1. *Zoospore before penetration of root hair.* ×*1366.* FIG. 2. *Stained zoospore in cortical cell shortly after penetration.* ×*780.* FIG. 3. *Two zoospores in root hair.* ×*1200.* FIGS. 4 AND 5. *Early stages in growth of zoosporangia in epidermal root cells.* ×*1300.* FIGS. 6 AND 7. *Development of lobes of zoosporangia.* ×*660.* FIG. 8. *Zoosporangium in epidermal cell.* ×*925.* FIG. 9. *Zoosporangium, with round barrel-like lobes.* ×*680.* FIG. 10. *Zoosporangium with greatly elongated lobes.* ×*688.* *All except Fig. 2, photographs of living fungus.*

Fig. 10). There are indications that a thallus of this type can pass through the walls of the host cell and invade neighbouring cells. A slight constriction occurs at the point of passage through the cell wall, and further development proceeds in the new cell.

The discharge tubes of the zoosporangium develop in the same manner as the lobes of the thallus. Short, blunt outgrowths grow toward the exterior wall of the host cell (Plate II, Figs. 11, 15 and 16). If the zoosporangium lies in an epidermal cell next to the surface of the root, a small round knob is formed which presses tightly against the cell wall. When the zoospores are formed a tiny opening appears in the centre of this knob through which they escape (Plate II, Fig. 20). Should the zoosporangium lie deeper in the cortex

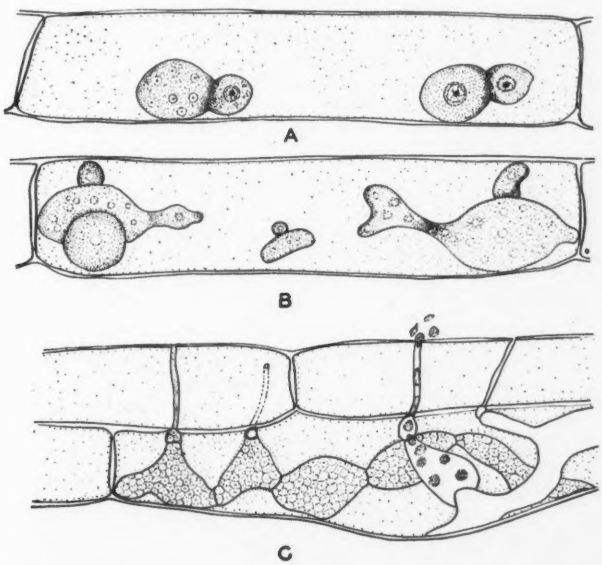

FIG. 1. *Polymyxa graminis.* FIG. 1A AND 1B. *Types of lobular outgrowths in early stages of zoosporangial development.* ×*600.* FIG. 1C. *Mature zoosporangia with discharge tubes passing through neighbouring host cells to reach the surface of the root.* ×*600. All figures drawn with the aid of the camera lucida.*

of the root, the discharge tubes may have to pass through adjacent cells to reach the exterior. A slight constriction appears at the point where the discharge tube passes through the cell wall and the tube remains long and narrow right to the surface of the root (Fig. 1C; Plate II, Fig. 19). Usually there is one discharge tube on each lobe of the zoosporangium, but occasionally there may be more (Plate II, Fig. 18). Viewed from the side the tube is cylindrical, and a septum is present at the point of connection with the zoosporangium, and also near the point of exit (Plate II, Fig. 16).

The question of whether the septa in the zoosporangium between the different lobes are true or pseudosepta can be determined most readily in zoosporangia that have discharged most of their zoospores. Zoospores trapped in the zoosporangium are confined to a single lobe and cannot pass through to another part of the thallus; also various lobes discharge their zoospores at different times. Therefore, the septum must be a true one. In the discharge tubes, however, openings occur in the septa to permit the passage of zoospores.

It is difficult to describe clearly all the changes which occur in the protoplasm of the thallus at the various stages of growth. Early in the course of development it is often finely granular and quite vacuolate in appearance (Plate I, Figs. 5 and 7). Some of the various changes in granularity, vacuolation and refringency of the protoplasm may be compared in the living thalli by examining Plate I, Figs. 3–10 and Plate II, Figs. 13, 15, 16, and 19. The fine refringent granules of a developing zoosporangium are clearly shown in Plate II, Fig. 15. The difference in appearance of a zoosporangium before and after zoospore delimitation is illustrated in Plate II, Fig. 13.

The walls of the empty zoosporangia tend to persist after the zoospores are discharged and later are frequently found in cells in which resting spore clusters have developed.

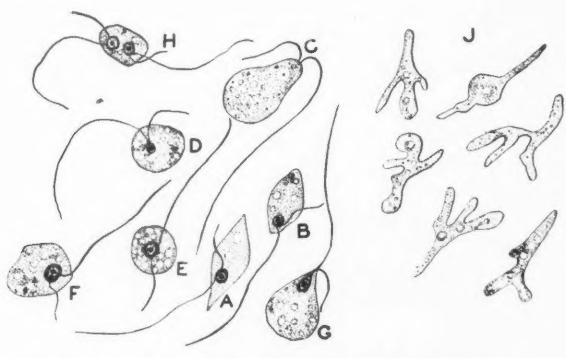

FIG. 2. *Polymyxa graminis.* A, B, C, D, E, AND F. *Zoospores illustrating some of the changes in shape.* × *1750.* FIG. 2H. *Binucleate spore with four flagella probably formed as a result of fusion of two uninucleate zoospores.* ×*1000.* FIG. 2J. *Amoeboid zoospores.* ×*1000. All figures drawn with the aid of the camera lucida.*

The Zoospores

The zoospores are fully developed within the zoosporangium and exhibit considerable movement even before they are discharged. Each zoospore oozes out of the end of the tube and appears at first as a small bubble on the surface of the host cell. Since there is no vesicle formed each zoospore is mature as soon as it leaves the discharge tube. When first released the zoospore is bulged in the centre with pointed ends (Fig. 2, A and B) and appears to be amoeboid for a moment; then the flagella begin to lash around and it

PLATE II. *Polymyxa graminis.* FIGS. 11 AND 12. *Zoosporangia; stained lacto-phenol acid fuchsin.* ×*300.* FIG. 13. *Zoosporangia; thallus on left, before zoospore formation; on right, zoospores nearly ready to be discharged.* ×*455.* FIG. 14. *Zoosporangium, indicating multinucleate condition, nuclei on right side in division. Stained lacto-phenol acid fuchsin.* ×*300.* FIG. 15. *Young zoosporangium, living.* ×*455.* FIG. 16. *Living zoosporangium showing side view of a discharge tube.* ×*660.* FIG. 17. *Surface view of zoosporangium showing exit tubes as small knobs underneath host cell wall; stained in lacto-phenol acid fuchsin.* ×*266.* FIG. 18. *Numerous exit tubes of a zoosporangium; stained in lacto-phenol acid fuchsin.* ×*266.* FIG. 19. *Discharge tubes penetrating through walls of adjacent cell to reach exterior of the root.* ×*1300.* FIG. 20. *Discharge tubes opening on cell wall; fixed in Flemming's weak fixer, unstained.* ×*1366.*

swims away with a rolling, turning motion. Should the zoosporangium lie deep in the cortex the zoospores will have to traverse the long slender discharge tube to the exterior (Fig. 1C). In such circumstances the zoospores pass along singly as in the mycelium of *Aphanomyces*.

From the work of previous investigators of the Plasmodiophorales, it was to be expected that the zoospores would be uniflagellate. Such appeared to be the case on first examination of living spores. However, it was noticed that even with the flagellum trailing, the zoospore was able to move rapidly, and the agitation among the bacteria and particles in the water at the anterior end of the spore close to the base of the long flagellum indicated that something was moving in that region. When stained preparations were made, proof was obtained of the presence of a short flagellum of about one quarter the length of the long one.

Most students of the lower fungi are agreed that zoospore flagellation is a very important character, which remains constant within a particular group. It was surprising, therefore, to fine one member of the Plasmodiophorales differing from the others in this respect. Accordingly, a re-investigation of the zoospores from the resting spores of *Plasmodiophora brassicae* and *Spongospora subterranea* was made, and they, too, were found to be biflagellate with a long and a short flagellum (8). The same character was found in the zoospores from the zoosporangia of *Spongospora subterranea* when these were discovered in 1935 (9), and just recently also for zoospores from spore balls of *Sorosphaera Veronicae*. This type of flagellation is quite different from that of any of the lower fungi or Myxomycetes and is not unlike that of heterokont algae (15). It is interesting to note that the short flagellum was also overlooked for many years in the algae, and its discovery caused a reclassification of the Chlorophyceae.

The living zoospore may be studied most conveniently in a very thin film of water on a cover slip over a Van Tiegham cell. The speed of swimming is thereby greatly reduced, making it possible to observe flagella, vacuoles and even the living nucleus. Under this very unfavourable environment it is not long before the zoospores encyst or become amoeboid. The period of zoospore activity depends largely on the environment, temperature being one of the most important factors. At 28° C. many zoospores become amoeboid and encyst in a few minutes, whereas at 18° C. they swim actively for several hours. If the zoospore is not in rapid motion it is generally spherical in shape. At times it may be top-shaped, pear-shaped, or ovoid (Fig. 2, A–E). Zoospores have been observed swimming with the flagella in front of the spore but usually they are propelled from behind. The body of the zoospore rotates over and over when in motion. If it is not very active it may rotate in a circle, pivoting around the long flagellum, which moves with a slow undulating movement, even when the zoospore is motionless.

After the zoospores have been swimming two or three hours under favourable conditions, or much sooner if the temperature is high, most of them become amoeboid. The flagella disappear and the amoebae move on the

surface of the substratum by means of pseudopodia (Fig. 2J). These amoebae vary greatly in shape and size. Usually they are very vacuolate, and the protoplasm in the pseudopodia has been observed to flow around and engulf small objects. It may be possible that they can ingest solid food particles in this way, in the same manner as that described by Maire and Tison (10) for zoospores of *Ligniera junci*.

Zoospores that have been fixed over osmic acid fumes, dried on a slide and stained, are spherical in shape. The average diameter of 150 stained zoospores was 4.2μ, with a minimum of 2.5 and a maximum of 5.6μ. The long flagella are 16 to 20μ in length and the short ones 4 to 5μ. A stained zoospore has finely vacuolate cytoplasm (Plate III, Fig. 24) with dark granules scattered throughout. Frequently a large dark spot in the centre of a clear area is visible on the margin of the zoospore. This may be a vacuole. The nucleolus of a zoospore nucleus is not as conspicuous as that of the amoeba within the host cell. The nucleus is usually ovoid (Plate III, Fig. 24), but may occasionally be top-shaped (Fig. 2G) or perfectly spherical (Fig. 2, E).

The flagella may be traced to the surface of the nucleus in stained material. They have sometimes been found to be attached close together at the base (Fig. 2D), though more often they are separated (Fig. 2, E and F). Although they appear occasionally to arise from the opposite poles of the nucleus, it is believed in these instances that the densely stained nucleus may mask the true proximity of the points of origin. Occasional zoospores with four flagella have been found (Plate III, Fig. 27). Such spores are invariably binucleate (Plate III, Fig. 28). It has not been possible to determine whether they are the product of a fusion between two zoospores or the result of incomplete separation within the zoosporangium.

The process of penetration by a zoospore has not been observed, although many attempts have been made to do so by transferring zoospores to young wheat rootlets growing in water or nutrient solution. One zoospore which had become amoeboid on the surface of a root hair was watched for 12 hours, but it did not penetrate (Plate I, Fig. 1). During this time several others had passed into nearby root hairs and assumed a spindle-shaped amoeboid form inside the host cell. No empty membrane could be found on the surface of the root hair after penetration of the zoospore as is the case with *Olpidium*. It may, therefore, be assumed that the entire amoeba passes into the cell as described by Curtis (5) for *Synchytrium*.

The Resting Spore Clusters

The resting spore thallus begins development in the host cell as a naked amoeba, and at no stage of development is a universal membrane present. Repeated nuclear divisions occur and a multinucleate myxamoeba is finally formed. Its spread being uncontrolled within the host cell, this myxamoeba may assume many different forms. Frequently it is long and tenuous, extending the whole length of the cell in which it started growth (Plate III, Fig. 30), or it may form a crescent-shaped mass about the host nucleus. At

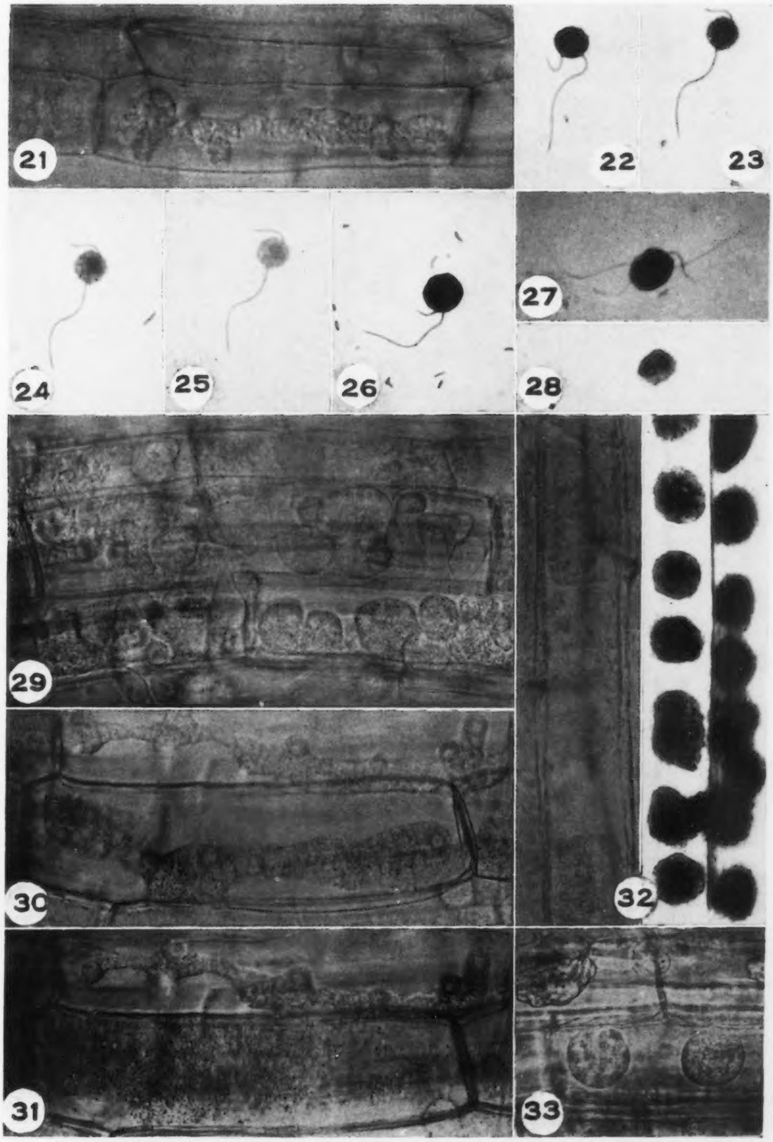

PLATE III. *Polymyxa graminis.* FIG. 21. *Mature zoosporangium with zoospores.* ×266. FIGS. 22–25. *Zoospores from zoosporangia, stained.* ×1200 FIG. 26. *Zoospore from resting spore, stained.* ×1366. FIG. 27. *Binucleate spore with four flagella, stained.* ×1200. FIG. 28. *Same spore as Fig. 27, showing nuclei, stained.* ×1200. FIG. 29. *Zoosporangium; also meronts that will form resting spores.* ×455. FIG. 30. *Naked myxamoebae, living.* ×455. FIG. 31. *The same thallus as in Fig. 30, but 20 min. later.* ×455. FIG. 32. *Meronts lying in tracheal cells; left, living; right, stained in lacto-phenol acid fuchsin.* ×455. FIG. 33. *Same stage as in Fig. 32 but in cortical root cell.* ×455.

this stage, long thread-like pseudopodia frequently radiate in various directions. Later these pseudopodia are retracted, the protoplasm of the fungus becomes much denser and the thallus may split up into a group of daughter cells or meronts, as termed by some writers. These meronts lie in rows or in closely packed groups in the tracheal and cortical cells (Plate III, Figs. 29, 32 and 33; Fig. 3, B and D). Fusions in the living fungus were occasionally observed among separate thalli lying in the same cell. Whether these were thalli of opposite sex or merely meronts from the division of a single mother

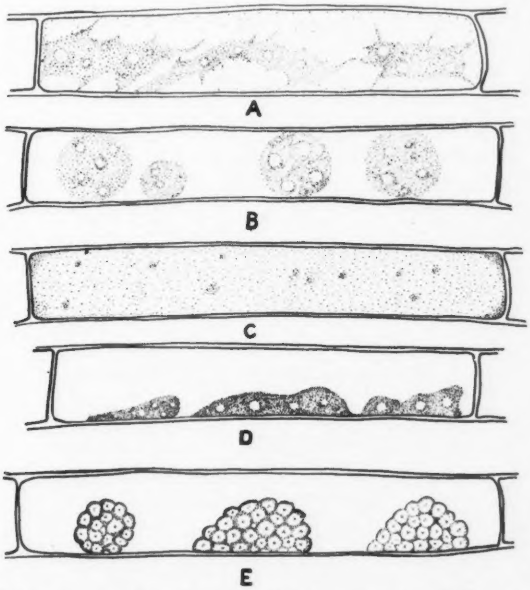

FIG. 3. *Polymyxa graminis.* FIG. 3A. *Naked myxamoebae during period of active growth.* ×*600.* FIG. 3B. *Meronts formed by division of myxamoebae.* ×*600.* FIG. 3C. *Same cell as in* 3B, *after the different meronts have coalesced.* ×*600.* FIG. 3D. *Myxamoebae just prior to the beginning of segmentation.* ×*600.* FIG. 3E. *Segmentation of myxamoebae to form resting spore clusters.* ×*600.*

thallus was not known. In one instance the granular protoplasm of four thalli kept milling slowly around until suddenly their contents spread out and fused into one mass, which filled the lumen of the host cell (Fig. 3, B and C). In another instance an elongate mass of naked protoplasm (Plate III, Fig. 30) suddenly spread out evenly in the host cell (Plate III, Fig. 31). The reason for the above phenomenon is not clear at present but is mentioned because it may have some significance in nuclear fusion.

During early stages of growth the myxamoeba appears very vacuolate, but as further development takes place the vacuoles gradually decrease in size and the thallus finally becomes a closely packed mass of dull granules.

(Plate III, Figs. 29 and 33). The density increases further and oily substances appear, which increase the refringency of the thallus just prior to spore delimitation (Plate IV, Fig. 39). Lines of cleavage appear around each nucleus, and by degrees cell walls are laid down, each consisting at maturity of an inner hyaline layer and an outer, smooth, brownish-yellow wall, which is fused with the neighbouring cells (Plate IV, Figs. 37 and 38). Most of the cells are quite spherical, but pressure exerted on the sides may cause some to be flattened or many-sided (Plate IV, Figs. 37 and 40). Individual cells of the spore clusters measure from 5 to 7μ in diameter. The contents are smooth and refringent without conspicuous oil droplets (Plate IV, Fig. 38).

It scarcely seems necessary to analyse the shape and size of the spore clusters in relation to those reported in the other genera as Palm and Burke (13) have done for *Sorosphaera Veronicae*, but some comparisons may be useful. Most of the spores are similar to those reported for *Ligniera junci* by Cook (1). Occasionally, individual spores similar to those of *Plasmodiophora brassicae* may be found (Plate IV, Fig. 36). Four spores grouped together as in *Tetramyxa* have been observed, and other clusters sometimes resemble the spore cakes of *Sorodiscus*, except for the absence of the universal membrane. The size and shape of the spore clusters and the great numbers present in heavily infected roots are shown in Plate IV, Figs. 35 and 36. Practically every cortical cell and even some of the tracheids in such roots are filled with spore clusters. Sometimes longitudinal rows of cells are infected (Plate IV, Fig. 34). This indicates that the fungus has been transferred from cell to cell during multiplication by division of an original infected meristematic host cell. There is no evidence that the myxamoeba can pass from cell to cell in non-meristematic host tissue.

Germination of the spores has been obtained on several occasions, but only after a prolonged treatment of alternate wetting, drying, and freezing. When zoospores appeared in a hanging drop of water containing a number of spore clusters, it was apparent from comparison of number and diameter of zoospores with empty cells in the spore cluster, that only one zoospore was produced by each cell. In manner of swimming and type of flagellation these zoospores were identical with those from the zoosporangia. Other writers have used the term swarm cells or swarm spores for these zoospores, as this is the terminology used in the Myxomycetes, but it seems preferable to discontinue this usage now, as the zoospores of the Plasmodiophorales are quite different from those of the Myxomycetes.

Cytological Studies

In all the species of Plasmodiophorales studied cytologically, nuclear behaviour during growth of the myxamoeba has been found to differ from ordinary mitosis. During the period of vegetative growth of the myxamoeba the conspicuous nucleolus persists and divides simultaneously with the chromatin within the nuclear membrane. On account of the unusual configurations, which at the metaphase take the form of a cross, it has sometimes

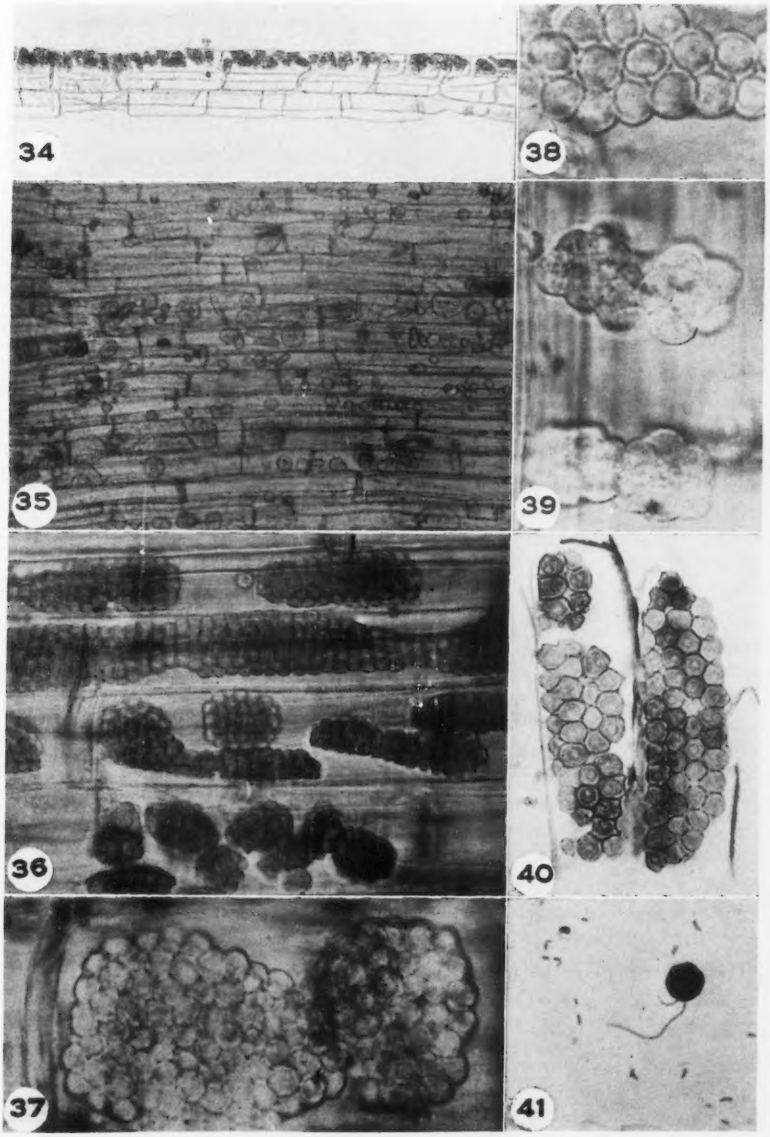

PLATE IV. *Polymyxa graminis.* FIG. 34. *Living spore clusters lying in one row of epidermal cells.* ×*90.* FIG. 35. *Strip of epidermal root tissue showing density of infection and varied shapes of spore clusters, stained, lacto-phenol acid fuchsin.* ×*405.* FIG. 36. *Spore clusters, stained lacto-phenol acid fuchsin.* ×*468.* FIG. 37. *Two characteristic spore clusters, living.* ×*925.* FIG. 38. *Individual cells of a spore cluster showing thickness of wall, living.* ×*1366.* FIG. 39. *Stage in development of spore cluster, walls just being formed, living.* ×*1366.* FIG. 40. *Section through spore clusters, stained safranin gentian-violet.* ×*1200.* FIG. 41. *Zoospore produced on germination, stained.* ×*1200.*

been called "cruciform division" but is more commonly known as protomitosis. Since this phenomenon has been observed frequently in the Plasmodiophorales and not elsewhere among the fungi, Cook (2) has suggested that the presence of protomitosis might be used as a diagnostic character of the group. Although it is not proposed to present a detailed account of the cytology of the fungus described in this paper, sufficient evidence will be presented to show that it is not unlike other members of the order in its nuclear behaviour.

The Somatic Nuclear Divisions in the Myxamoebae

The first indication of nuclear division in the myxamoeba is an enlargement of the nucleus and an increase in the intensity of staining of the chromatin granules, which radiate out from the nucleolus like the spokes of a wheel. Gradually a ring of chromatin appears and in polar view it completely surrounds the nucleolus. At right angles to the polar axis the ring appears as a band across the nucleolus, but if viewed at a slightly different angle the latter may lie slightly above or below it (Plate V, Figs. 45 and 48). This configuration is similar to that called the "balance scale" by Nawaschin (11), or the "unipolar spindle configuration" by Horne (6). No asters or centrosomes were found at this stage as reported by Nawaschin in his studies. The nucleolus now elongates and projects through the ring of chromatin as a short blunt peg. Further elongation of the nucleolus produces the typical "cruciform" figures which have so often been described by the various workers on the cytology of the Plasmodiophorales (Plate V, Figs. 44–48). Apart from the presence of the nuclear membrane and the division of the nucleolus with the chromatin, this is in reality a ring-like metaphase figure and has been described as such by Horne (6). The chromatin was distinctly granular in all rings examined, and in some cases was organized into more or less definite bodies believed to be chromosomes. The next step in division involves splitting of the ring into two halves that move apart toward the ends of the cylindrical nucleolus. Horne (6) calls this the telophase. It corresponds to the "double anchor" or "dumb-bell" figures of other writers (Plate V, Figs. 49 and 50). Next the two halves of the nucleolus contract and separate completely, the spindle fibres disappear, and the nuclear membrane, which has been present throughout the whole process of division, constricts and closes in around the daughter nuclei. Thus through repeated divisions of this type a multinucleate thallus is developed. There is remarkable uniformity within a single myxamoeba in the time of occurrence of the different phases of nuclear division.

The Transitional Phase

The nucleoli disappear when the myxamoeba has attained its full growth, and simultaneously densely staining granules appear in the cytoplasm surrounding the nuclei. This has been called the akaryote stage by Cook (2), Osborne (12), and Schwartz (14), and the transitional phase by Horne (6) and Webb (17). Although earlier students thought the chromatin was

also extruded, leaving an empty nucleus, Webb (17) has demonstrated that this is not true, because a fine chromatin reticulum may be seen within the nuclear membrane if Newton's iodine-gentian-violet method of staining is employed. Webb's observation has been substantiated in the present study. In preparations stained with iron-alum haematoxylin the nuclei appeared to be devoid of chromatin, whereas iodine-gentian-violet revealed its presence. In *Sorosphaera Veronicae*, Webb has described a nuclear fusion during this phase; but although nuclei have been found associated in pairs, it was impossible in the present study to determine whether actual fusion took place.

Nuclear Divisions Preceding Spore Formation

By analogy with other species of Plasmodiophorales, meiosis should occur in the divisions that precede the segmentation of the myxamoebae into the spores that make up the spore clusters. So far it has been impossible to count the number of chromosomes present in the nucleus, so definite proof of meiosis must await further studies. In division figures found at this stage of development of the fungus, both the nucleolus and the nuclear membrane had disappeared, and the chromatin formed prophase, metaphase, anaphase, and telophase configurations (Plate V, Figs. 52–57) of the ordinary type found in higher plants.

Nuclear Divisions in the Zoosporangia

Throughout the whole period of growth of the zoosporangium there is no division of the nucleolus with the chromatin within the nuclear membrane comparable to that observed in the growing myxamoeba. All divisions were of the ordinary mitotic type, and even in the so-called resting nucleus no conspicuous nucleolus was present. These observations are in agreement with those made by Cook and Schwartz (4) on the nuclear divisions in the zoosporangia of *Plasmodiophora brassicae*, except that they state that the resting nucleus resembles in all essential features the resting nucleus of the plasmodium. In *Ligniera junci*, however, Cook has described protomitotic nuclear division in early stages of zoosporangial formation.

Discussion of Systematic Position

In considering the affinities of *Polymyxa graminis* with other non-filamentous intracellular parasites, it is obvious that the character of the resting spores alone separates it from the Synchytriaceae, Woroninaceae, Olpidiaceae and Ancylistaceae. Superficially the zoosporangia bear some resemblance to those of *Septolpidium lineare*, described by Sparrow (16) in 1933, since their course of development is similar, and at maturity they consist of a series of conjoined truncated segments. However, the fact that *Septolpidium* is an algal parasite with uniflagellate zoospores, which form a vesicle when discharged through non-septate discharge tubes, indicates at once that it is not closely related to *Polymyxa*.

Several lines of evidence, such as the type of resting spore clusters that develop from multinucleate myxamoebae, the method of nuclear division during

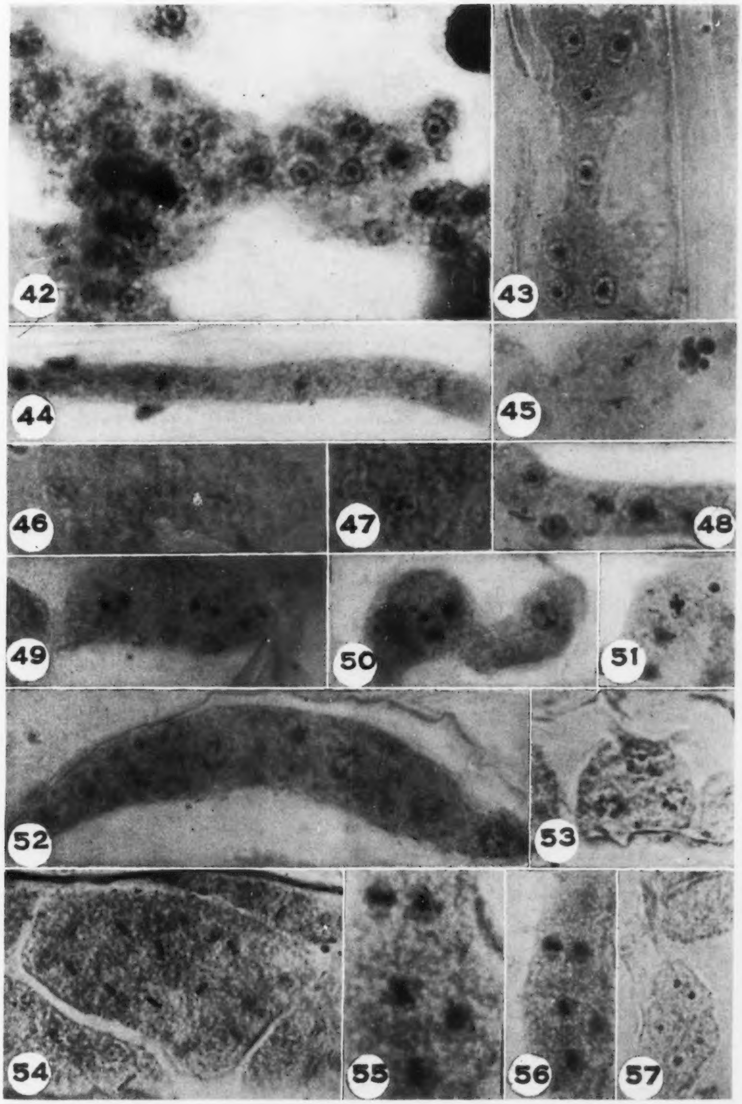

PLATE V. *Polymyxa graminis.* FIG. 42. *Multinucleate myxamoeba stained in Haidenhain's haematoxylin without sectioning root.* ×*1375.* FIG. 43. *Young myxamoeba. Note absence of a definite wall. Stained in safranin gentian-violet.* ×*1140.* FIGS. 44–48. *Metaphase plates of protomitotic division, stained in safranin gentian-violet.* FIGS. 49 AND 50. *Telophase figures of protomitotic divisions.* FIG. 51. *Metaphase plate of protomitotic divisions stained by Newton's iodine gentian-violet method.* ×*1300.* FIGS. 52 AND 53. *Mitotic prophase.* ×*1366.* FIG. 54. *Metaphase plates.* ×*1366.* FIG. 55. *Metaphase plates.* ×*2050.* FIG. 56. *Metaphase plate, stained in Haidenhain's haematoxylin.* ×*1366.* FIG. 57. *Telophase.* ×*1366.*

growth of the myxamoeba, and the characteristic biflagellate zoospores, point to relationship with the Plasmodiophorales. Generic differences in this order have hitherto been made almost entirely on the arrangement of the mature resting spores. The presence of spore clusters in *Polymyxa* separate it from *Plasmodiophora*, in which the spores are free. Inclusion with either *Sorodiscus* or *Sorosphaera* is not justified, because the spores in these genera are grouped into flat spore cakes or spore balls which are surrounded by a universal membrane. Likewise the spongy spore balls of *Spongospora* or the tetrad-like groups of spores in the genus *Tetramyxa* are quite different. Only with *Ligniera*, in which the spores are arranged in aggregates of indefinite size or shape, is there marked similarity of resting spore characters, but the dissimilar zoosporangia and absence of schizogony in this genus preclude the inclusion of *Polymyxa*.

Until quite recently zoosporangia were not thought to be present in the Plasmodiophorales. Cook (1) was the first to describe them in *Ligniera junci*, followed by Cook and Schwartz (4) for *Plasmodiophora brassicae*, and the writer (9) for *Spongospora subterranea*. In these three species the zoosporangia are small simple structures lacking zoospore discharge tubes. In *P. brassicae* and *S. subterranea* they are very evanescent bodies usually present in the root hairs of the host. Although these zoosporangia appear to be quite different from the conspicuous, highly organized type present in *Polymyxa*, when direct comparisons were made with *Spongospora subterranea*, the zoospores produced from each were found to be almost identical. In fact it is the presence on such zoospores of two flagella, one long, the other short, that provides the best evidence for close relationship between these species, even though there is considerable difference between the two species in zoosporangial and resting spore characters. In this respect the Plasmodiophorales are by no means unique, for it is a well known fact that within the different families of lower fungi, zoospore characters are quite similar in different species and genera of a single family, whereas there is frequently considerable variation in the zoosporangia and resting spores. The practice has generally been to use these latter differences for the separation of genera and species. Thus in the Olpidiaceae such characters as the location of the sporangia in the host cells, the presence or absence of exit tubes on the zoosporangia, and companion cells on the resting spores, are of importance. Indeed, the zoosporangia provide most of the important characters used to separate genera in several families of the lower fungi. There is ample precedence therefore to place more emphasis on zoosporangia in classification of the Plasmodiophoraceae. Most of the present difficulties in the taxonomy of this family are due to lack of knowledge of complete life cycles, thus making it necessary to place too much emphasis on minor differences in resting spores as a means of separating genera. Palm and Burke (13) have even suggested that it might be preferable to merge all the present genera—with the exception of *Cystospora*, which is a doubtful member—into the genus *Plasmodiophora*. It seems to the writer that this proposal only transfers the difficulties

encountered in separating genera to the species, without improving the present system of classification. For the present it appears advisable to retain the six genera recognized as valid by Cook (3) in his monograph of the Plasmodiophorales, adding to these the new genus *Polymyxa* with the following diagnosis.

Diagnosis

Polymyxa n. gen.

Resting spore clusters of indefinite size and shape, without universal membrane; produced by schizogony and segmentation from naked multinucleate myxamoebae. Zoosporangia conjoined series of thin-walled, lobular, multinucleate segments, each with one or more discharge tubes. Similar zoospores produced by resting spores and zoosporangia, each bearing two flagella, one long and one short.

Polymyxa graminis n. sp.

Resting spores spherical or many sided, 5 to 7μ in diameter, smooth, yellow-brown outer walls. Zoosporangia large, septate, with persistent, smooth, thin walls. Discharge tubes segmented. Zoospores numerous, 4 to 5μ in diameter, discharged without vesicle formation. Non-hypertrophying, obligate parasite, in roots of *Triticum aestivum* L., *T. durum* Desf., *Hordeum vulgare* L., and *Secale cereale* L.

Type specimens are deposited in the herbarium of the Botany Department, University of Toronto, Ontario.

Polymyxa genus novum

Glomerulis sporarum quiescentium magnitudine et forma indefinitis, sine membrano universali, per fissionem sejunctionemque e nudis multinucleatis myxamoebis generatis. Zoosporangiis in seriem tenui-tunicatorum, lobulatorum, multinucleatorum segmentorum conjunctis, germinatione zoosporas per unum vel plures processus evacuantibus. Zoosporae e sporis quiescentibus formatae sunt similes illis e zoosporangiis exeuntibus; singulae zoosporae duo flagella, unum longum, sed alterum breve, ferent.

Polymyxa graminis species nova

Sporis quiescentibus globosis vel multilateralibus, levibus, fulvis, 5 to 7μ diam. Zoosporangiis magnis, septatis, cum muris persistentibus levibus tenuibus. Processibus evacuationis in segmenta divisis. Zoosporis copiosis, 4 to 5μ diam., sine origine vesicarum emissis. Thallo parasitica obligato, non tumefaciente in radicibus matricis.

In radicibus Tritici aestivi L., T. duri Desf., Hordei vulgaris L., et Secalis cerealis L.

Acknowledgments

It is a pleasure to express my gratitude to Professor D. L. Bailey and Professor H. S. Jackson of the Botany Department, University of Toronto, for supervision, suggestions, and kindly criticism during the course of this study.

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THE EFFECT OF SALICYLIC ALDEHYDE ON THE INFECTION OF WHEAT BY *PYTHIUM ARRHENOMANES* DRECHSLER, AND THE DESTRUCTION OF THE ALDEHYDE BY *ACTINOMYCES ERYTHROPOLIS* AND *PENICILLIUM* SP.[1]

By V. E. Graham[2] and L. Greenberg[3]

Abstract

Salicylic aldehyde, when added to soil at the rate of 50 p.p.m., seems to predispose wheat roots to attack by parasitic strains of *Pythium arrhenomanes*. *Actinomyces erythropolis* and a species of *Penicillium* have been found in soil from the healthy area of a field partially infected with Browning root rot. These organisms caused the disappearance of salicylic aldehyde in an artificial medium. It is suggested that lack of activity on the part of such organisms in certain areas of a field may lead to an accumulation of salicylic aldehyde or products acting in a similar manner, and that this may be a predisposing factor in the appearance of Browning root rot caused by *Pythium arrhenomanes*.

Introduction

During an investigation of host-conditioning factors that influence the severity of root rot of sugarcane by *Pythium arrhenomanes*, Rands and Dopp (3) found that the presence of salicylic aldehyde in the soil increased the susceptibility of the cane to fungus attack. Concentrations of salicylic aldehyde of 20 and 40 p.p.m. parts of soil had little influence on cane growth in the absence of the fungus, but when the fungus was present an apparent predisposition of the roots to attack became evident. A reduction in weight of plants was obtained that amounted to two to seven times the reduction found when only the fungus was present. The prevalence of *Pythium* root rot of sugarcane on the heavy, poorly drained soils of Louisiana was thought to be due, in part at least, to the presence of this and similarly behaving compounds. Improvement in drainage and general fertility in such areas was reported to increase markedly yields of cane.

Shorey (4) showed that salicylic aldehyde was present in some soils and attributed its presence to the addition of the aldehyde itself or to bodies yielding it in vegetative remains. Skinner (5) mentioned that aldehyde (not necessarily salicylic aldehyde) was more commonly found in acid soils, but that it was also obtained in certain cases from neutral and alkaline soils. He found that salicylic aldehyde was not harmful to the crops tested in culture solutions in amounts of 10 p.p.m., but that 25 p.p.m. was decidedly harmful. In soils, 50 p.p.m. was injurious to corn in both sand and clay soils, the effect being more marked in sand. Soils with strong oxidizing power (as determined by the oxidation of aloin) overcame the effect of the aldehyde, and the theory was advanced that the destruction of the added aldehyde was due to biological processes. In the soils tested, the effect of the added salicylic

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aldehyde was overcome by the application of lime. Phosphorus also reduced the damage in all cases and eliminated it entirely in some. Field experiments verified these results, and indicated that the aldehyde persisted in some soils and disappeared in others and that this could be linked to the oxidizing power of the soil. The soils in which the aldehyde was destroyed had strong biological activities, good oxidizing powers, and were fertile. Salicylic aldehyde was found to occur more frequently in soils of low productivity. No mention is made in any of these studies of disease having an influence on the results obtained. In solution cultures, however, it was found that the addition of 10 p.p.m. of manganese completely overcame the adverse effect of the added aldehyde and that the roots were less stunted in the presence of lime.

Browning root rot of wheat in Saskatchewan is caused primarily by *Pythium arrhenomanes* (7). There has been observed, in central and northern Saskatchewan, a tendency for the diseased patches of a field to occupy slightly depressed positions as compared to the uninfected portions. The moisture content of the soil in these Browning areas is usually higher than that of corresponding disease-free areas of the same field. This may be explained partly or wholly by the lowered demand on soil moisture due to the stunted growth. On the other hand, it is not uncommon to find fields of rolling topography in which the wheat plants are healthy on the higher and lower areas and infected on areas of intermediate elevation. This condition is not comparable to a heavy, poorly drained soil as mentioned by Rands and Dopp (3). The exact relation of drainage to the incidence of Browning root rot is not clear.

Experimental

Salicylic Aldehyde as a Predisposing Factor to Pythium Damage

The results obtained by Rands and Dopp (3), Shorey (4), and Skinner (5), suggested the need for a study of the effect of salicylic aldehyde on the severity of attack of wheat roots by *Pythium arrhenomanes*. To determine whether the aldehyde would predispose wheat to attack by the fungus the following experiment was devised. A weighed amount of soil (1500 gm.) was placed in each of eight pots. These were sterilized in the autoclave at 20 lb. pressure for four hours and then in the hot air oven at 160° C. for two hours. Duplicate pots were treated according to the following scheme:

A. Controls—not treated.

B. Salicylic aldehyde added to give a concentration of 50 p.p.m.

C. Salicylic aldehyde, 50 p.p.m. Inoculated with *Pythium arrhenomanes*, strain SH_2.

D. Inoculated with *Pythium* only.

Two days after treatment, 20 disinfected wheat seeds were planted in each pot at a depth of one inch. The fungus was grown on a mixture of sterile soil and cornmeal, and a blank of this medium was added to the other pots to eliminate any difference due to the cornmeal. The resulting crops produced

in the greenhouse were photographed when three weeks old and are shown in Fig. 1. The apparent effect of salicylic aldehyde in intensifying the injury produced by the fungus is evident in the illustration.

Isolation of Organisms That Decompose Salicylic Aldehyde

Skinner (5) referred to the disappearance of salicylic aldehyde from the soil and mentioned that this was due to biological processes. No mention was made of specific organisms capable of producing this result. Two such organisms were isolated by the following simple procedure. A nutrient solution was prepared that contained the salts used by Thornton (6) in counting bacteria in soil, but with the agar and mannitol eliminated. This

FIG. 1. *C—control, sterilized soil; SA—sterilized soil plus 50 p.p.m. salicylic aldehyde (Eastman Kodak Co.); P—sterilized soil plus inoculum of Pythium arrhenomanes; SA + P—sterilized soil plus 50 p.p.m. salicylic aldehyde plus inoculum of Pythium arrhenomanes.*

was sterilized in 200-ml. quantities in 500-ml. flasks. Salicylic aldehyde was added after sterilization to give concentrations of 10 and 20 p.p.m., respectively. This medium was inoculated with a suspension of organisms obtained by shaking up 25 gm. of soil from the healthy area of a partially infected field, allowing the coarser particles to settle, and withdrawing 0.1 ml. of the relatively clear suspension. After three weeks' incubation at room temperature, the material in the flasks was plated on Thornton's agar for bacteria and on peptone-glucose-acid agar for moulds. No moulds were obtained from the flasks containing 20 p.p.m. of aldehyde and only one type appeared on the plates from the 10 p.p.m. flasks. Sixteen cultures of bacteria were isolated from the plates.

The individual ability of each of these crude cultures to destroy salicylic aldehyde when grown on a mineral-salt–salicylic-aldehyde mixture was then tested. After 4 days' incubation at room temperature the salicylic aldehyde had disappeared from 4 tubes; after 11 days' incubation it had disappeared

from 2 more, making a total of 6 positive aldehyde-destroying cultures out of the 16 tested. The aldehyde had not been destroyed by the mould culture in 4 days but was destroyed after 11 days. Through the courtesy of Dr. J. E. Machacek, of the Dominion Laboratory of Plant Pathology, Winnipeg, the mould culture has been tentatively identified as belonging to the *Penicillium janthinellum* series and is probably *P. Rivolli Zaleski*.

Microscopic examination of the bacterial cultures indicated that there was a Gram-positive branching organism present in all those that caused the disappearance of salicylic aldehyde. This organism apparently belonged to the *Actinomycetaceae* (Bergey (1)). Culture No. 7 was a pure culture of this organism, and it has been identified as *Actinomyces erythropolis* (Gray and Thornton) (2), (Bergey) (1). This organism was originally isolated by Gray and Thornton (2), who reported that it caused the breakdown of phenol and m-cresol. A comparison of Culture No. 7 with the organism of Gray and Thornton proved that they are identical.

Discussion

This study of the effect of salicylic aldehyde when added to soil and its relation to the attack of wheat roots by *Pythium arrhenomanes* was commenced too late in the season to allow any field work to be done on the subject. The fact, however, that salicylic aldehyde was found in many soils by Skinner (5), coupled with the observation that the addition of this aldehyde to soil seems to increase the susceptibility of the roots of both wheat and sugarcane to attack by *Pythium arrhenomanes*, is considered to be very suggestive. It remains to be seen whether salicylic aldehyde can be isolated in larger quantities from diseased areas under natural conditions, than from unaffected soil. The effect of the salicylic aldehyde seems to be on the plant rather than on the fungus, as shown by Rands and Dopp (3) and also by our experiments in which it was proved that the fungus could not grow on a medium containing 10 p.p.m. of salicylic aldehyde—an amount which was without effect on wheat.

Vanterpool (7) has shown that the addition of phosphorus fertilizer to soil eliminates serious damage to wheat from *Pythium arrhenomanes*. Skinner (5) showed that the deleterious effect of salicylic aldehyde in soil was lessened by the application of phosphorus and that 10 p.p.m. of manganese overcame it entirely in solution cultures. These results are very interesting, and it is suggested that there should be determinations made of the amount of manganese in healthy and diseased soil and in phosphorus fertilizers. It is quite possible that other substances, acting somewhat as catalysts, would have an effect similar to that of manganese. A study of the effect of some of these substances on the growth of *Actinomyces erythropolis* is also suggested.

This preliminary study suggests that when soil conditions are such as to inhibit the growth of organisms that are capable of breaking down salicylic aldehyde, this substance will accumulate in the soil and contribute to the severity of the attack on the roots by *Pythium arrhenomanes*. It is realized

that other substances may be capable of predisposing wheat to attack in a similar manner. In any case, micro-organisms have been found in soil that can destroy salicylic aldehyde, although the manner in which the aldehyde is destroyed is not known. It is apparent that qualitative relations between the flora of infected and uninfected areas may be of importance in determining the incidence of the disease. This hypothesis affords a reasonable explanation of the tendency for Browning disease to appear in isolated areas of an otherwise healthy field.

Acknowledgments

Thanks are due to Professor T. C. Vanterpool, University of Saskatchewan, for supplying cultures of *Pythium arrhenomanes* and taking the photograph used in Fig. 1, and to Dr. J. E. Machacek, Dominion Laboratory of Plant Pathology, Winnipeg, for the tentative identification of the salicylic-aldehyde-decomposing fungus.

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COLOUR OF MEAT: II. EFFECT OF DESICCATION ON THE COLOUR OF CURED PORK[1]

BY C. A. WINKLER[2]

Abstract

In the absence of air, a linear relation, independent of temperature, was observed between moisture loss and colour change. The change was mainly one of intensity, and was reversible. In air, irrespective of its humidity, changes in both intensity and quality of colour occurred, but no definite relation was found between changes in humidity and colour quality for different samples. In saturated air, intensity changes became complete in the early part of the storage period, but the enhanced changes in air of lower humidity continued to increase. No influence of temperature on the rate of colour change was observed in dry air or in air of 60% relative humidity.

Introduction

The two more important factors contributing to the surface discolouration of meat are methaemoglobin formation and desiccation. The formation of methaemoglobin on the surface of bacon has received considerable attention, notably by Brooks (1), but the effect of desiccation on the colour of this meat seems not to have been quantitatively investigated. Some results that were obtained during a study of this problem are given in the present paper.

Apparatus

Colours were measured with the photoelectric colour comparator previously described (4). With this instrument the amount of light scattered at right angles to the surface of the sample in the blue, green, and red portions of the spectrum is measured and expressed as a percentage of the amount similarly scattered in the same spectral regions from a standard white surface under the same light intensity. The spectral regions are defined by standard colour filters in the path of the scattered light. The filters used in the present work transmitted the following wave lengths: Blue, 4000–4500Å; Green, 4900–5800Å; Red, 5750–7000Å.

Experimental Methods and Results

Colour Change by Vacuum Drying

Moisture is lost at a greater rate from the surface than from the interior of a slice of bacon when drying occurs. Therefore, to make a quantitative

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study of the relation between change in surface colour and amount of desiccation of sliced bacon, it is necessary either to assume that the moisture lost from the surface layer is directly related to the total moisture loss, or to use very thin slices. There is no reason to suppose the first alternative to be a valid assumption, and the second alternative is difficult to adopt experimentally. By the use of minced bacon, however, the average colour change corresponding to a given loss of moisture throughout the sample can be obtained, and minced bacon has therefore been used in several of the experiments to be described.

Weighed samples (about 50 gm. each) taken from the same batch of minced, unsmoked bacon were dried in *vacuo* at 0° C., 10° C., and 25° C. Each sample was periodically weighed, thoroughly mixed and its colour measured. A plot of the results for a typical experiment is shown in Fig. 1, from which it is evident that the relation between colour change and moisture loss is linear and independent of temperature. The change in colour is mainly one of intensity rather than quality; this is inferred from the fact that a decrease of about 43% in scatter of blue, green or red, corresponding to a 50% moisture loss, is accompanied by not more than 6% change in the ratios of scatters, *i.e.*, red/green, red/blue, or green/blue.

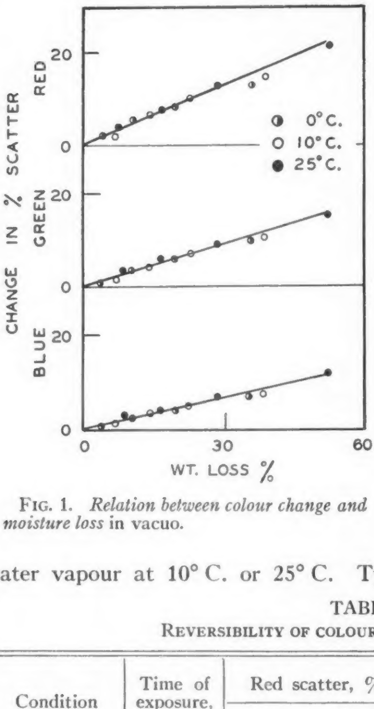

FIG. 1. *Relation between colour change and moisture loss* in vacuo.

The colour change caused by vacuum drying was found to be reversible when partially dried samples of sliced or minced bacon were placed in an atmosphere of water vapour at 10° C. or 25° C. Typical results are shown in Table I.

TABLE I

REVERSIBILITY OF COLOUR CHANGE AFTER DRYING *in vacuo*

Condition	Time of exposure, hr.	Red scatter, %		Green scatter, %		Blue scatter, %	
		10° C.	25° C.	10° C.	25° C.	10° C.	25° C.
Initial colour	–	34.0	33.4	24.2	24.2	22.0	21.2
Moisture loss	2	–	29.8	–	21.5	–	18.0
Moisture loss	12	26.0	27.8	19.0	18.9	18.6	17.8
Moisture gain	12	28.4	29.1	20.0	20.8	19.4	18.6
Moisture gain	24	31.2	–	21.3	–	20.4	–
Moisture loss	16	27.3	–	18.2	–	17.0	–
Moisture gain	54	–	31.0	–	22.5	–	20.2
Moisture gain	96	34.0	–	24.4	–	22.4	–

At 10° C. the reversibility was complete, providing the samples remained in contact with the water vapour a sufficient length of time. At 25° C., formation of bacterial slime on the surface of the meat always preceded complete recovery of the initial colour, otherwise the behaviour was similar to that observed at the lower temperature.

Colour Change in Air at Different Relative Humidities

In the vacuum drying experiments, the absence of oxygen precluded colour changes due to methaemoglobin formation. If, however, bacon is stored in air at 100% relative humidity, the formation of methaemoglobin should be the only factor responsible for colour change. On the other hand, the changes observed in air at lower relative humidities should represent the combined effects of drying and methaemoglobin formation.

Several experiments were made to determine the nature and relative amounts of the colour changes resulting from oxidation alone, and from oxidation and drying combined. For each experiment, duplicate samples of about 50 gm. each, taken from the same batch of minced, unsmoked bacon, were stored at room temperature in desiccators through which a slow stream of either saturated or dry air was passed. At intervals, each sample was thoroughly mixed and the amount of colour change determined. The results of a typical experiment are plotted in Fig. 2A.

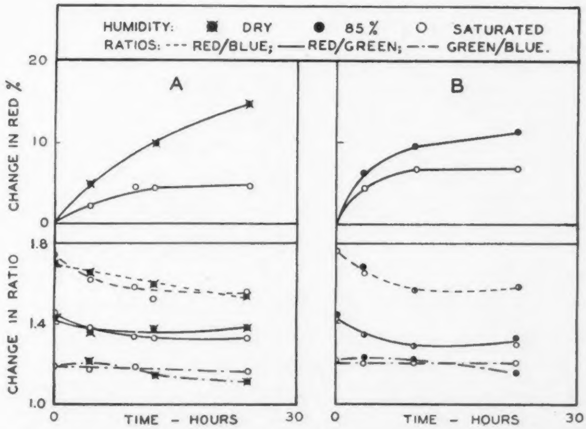

FIG. 2. *Effect of storage in dry and moist air on colour intensity and quality.*

Unlike storage *in vacuo*, storage in dry or saturated air produced marked changes in quality (upper curves) as well as in intensity of colour (lower curves). In air of 100% relative humidity, change in colour intensity, as inferred from change in red scatter, always attained a maximum value, presumably after methaemoglobin formation had gone to completion. In

dry air, the colour intensity continued to change throughout the storage periods used.

The quality of the colour developed in dry air usually differed somewhat from that developed in saturated air, for samples taken from the same batch of minced bacon. However, the results of a number of experiments with samples from different batches failed to indicate any definite relation between the quality of the colour and relative humidity. This is perhaps to be expected, as the rate of methaemoglobin formation probably depends upon both the rate and extent of drying (2), and these two factors will almost certainly vary from sample to sample.

While the experiments in dry and saturated air were satisfactory for determining the nature and relative magnitudes of the changes in colour of bacon stored under extreme humidity conditions, it was thought desirable to ascertain also the extent to which drying influenced the colour when the humidity approached that used in commercial practice. Experiments were therefore made in which samples of minced, unsmoked bacon were stored at room temperature in slow streams of air conditioned to 85% and 100% relative humidity. The results for a typical experiment are plotted in Fig. 2B. It is evident from the curves for both change in ratio and change in red scatter that during the first few hours' storage at 85% relative humidity, methaemoglobin formation is responsible for most of the observed colour change. Subsequently, however, the effect of drying on colour intensity becomes important, as shown by the increased change in red scatter. With a given sample of bacon, there is little difference in the quality of the colour developed during storage at the two humidity levels.

A few experiments were also made with slices of unsmoked and smoked bacon in air of 60% and 100% relative humidity at 10° C. The results obtained with both these meats in the sliced condition were similar to those shown in Fig. 2B, except that the effect of drying was more marked at the lower humidity and lower temperature. Changes in colour quality were again found to be similar at the two humidity levels.

Effect of Temperature

The effect of temperature on the rate of colour change under drying conditions was studied by storing 50-gm. samples of bacon, both smoked and unsmoked, in a slow stream of dry air at 10° C. and 25° C. Results for typical experiments are shown in Fig. 3. It is apparent that for a given moisture loss from each type of meat, the change in colour intensity is the same at both temperatures. Although slight differences in quality were observed, there was no apparent relation between changes in temperature and colour quality with different samples. Experiments were also made with slices of bacon stored in air of 60% relative humidity at 0° C. and 10° C. Again, no influence of temperature on the rate of colour change could be observed in the temperature interval used.

It is somewhat surprising that the part contributed to the colour change by methaemoglobin formation is not sufficiently different at the different temperatures to be revealed. Presumably the effects due to drying masked the discolouration resulting from methaemoglobin formation, since the changes observed in colour quality indicate that methaemoglobin was formed. It might well be that drying in air of higher humidity would permit a temperature coefficient of colour change to be detected. It might be mentioned,

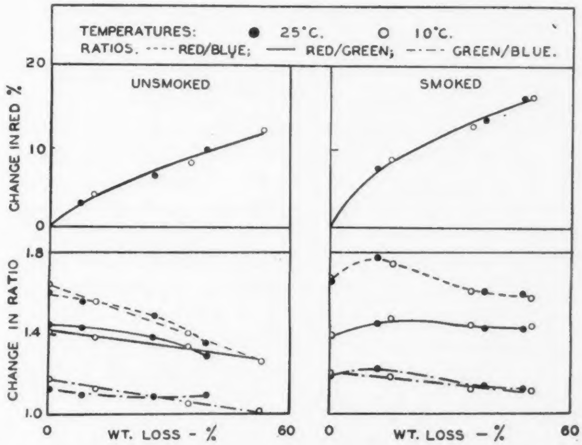

FIG. 3. *Effect of temperature on colour intensity and quality under drying conditions.*

however, that some preliminary studies of the influence of temperature on methaemoglobin formation in minced bacon stored in saturated air gave very variable results. With some samples, temperature appeared to have no influence, while with others the rate of methaemoglobin formation increased two to three times for an increase of 10° C.

Discussion

Quantitatively the results of this investigation are applicable only to small samples, but certain qualitative generalizations are also possible. When the results are considered as a whole it is evident that in the absence of air and under conditions that prevent desiccation, the colour of bacon should be subject to change only through putrefaction, a conclusion essentially similar to that arrived at by Brooks (1) from other experimental evidence. In the presence of either dry or moist air, however, methaemoglobin formation causes changes in both intensity and quality of the surface colour, the intensity changes but not the quality changes being enhanced by drying.

The effect of desiccation becomes increasingly significant as the storage period is prolonged. Methaemoglobin formation, however, seems to attain to

a maximum value, although a more sensitive instrument than that used might show a slow formation of methaemoglobin following the rapid initial change. According to Brooks (1), methaemoglobin formation is confined to a superficial layer about 5 mm. in thickness, but there is no reason to suppose that the effect of drying is similarly limited, since loss of moisture undoubtedly occurs from the body of the sample. Loss of considerable moisture from the interior of the meat, *i.e.*, in the absence of oxygen, might result simply in changes in the intensity of the internal colour, but there is some evidence (5) that loss of even a relatively small amount of moisture might have profound effects on the colour of freshly cut surfaces.

The reversibility of the changes in colour intensity caused by vacuum drying indicates that the meat pigments are not appreciably denatured by desiccation at ordinary temperatures, and the suggestion of Heiss and Hohler (3) that cathaemoglobin is formed during the drying process seems rather improbable. It seems unlikely also that these results, obtained with minced bacon, can be satisfactorily explained on the assumption that intensity changes caused by drying are due solely to optical effects (2). Concentration of the pigments by removal of water would seem to afford a more satisfactory explanation of both the observed changes and their reversibility.

Acknowledgment

Grateful acknowledgment is made to Mr. E. A. Rooke for valuable technical assistance throughout the investigation.

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DEW-POINT HYGROMETER FOR USE AT LOW TEMPERATURES[1]

BY C. A. WINKLER[2]

Abstract

An apparatus is described in which provision for slow cooling of a metal mirror by circulating over it liquid from a vessel in a thermoregulated bath, and the use of multiple thermocouple elements contained in the mirror, enable the dew-point temperature to be gradually approached and accurately determined. Precise measurements of relative humidity at low temperatures, where the moisture content of the air is small, are therefore possible. A precision of $\pm 0.5\%$ relative humidity was readily attained at temperatures down to $-15°$ C.

Introduction

Accurate measurement of relative humidity, as an essential aspect of its accurate control, is important in relation to the refrigerated preservation of perishable products. Although many instruments, based on several different principles, have been developed for the measurement of humidity (2), many of these are not sufficiently sensitive to permit precise estimation of the moisture content of air at low temperatures, while others of greater sensitivity are generally empirical and require calibration. There is, therefore, an obvious need for an instrument based on sound theoretical principles and capable of accurate determination of relative humidity at low temperatures. These considerations led to the development of the absorption and dew-point instruments described in the present paper.

Description of Instruments

Absorption Hygrometer

Attempts were made to develop an absorption hygrometer suitable for determining the humidity of air samples drawn from packaged products stored at low temperatures. The apparatus was designed to prevent both access of the sample of air to the desiccant during sampling, and volume changes in the system when the desiccant was brought into contact with the sample. In its final form, the hygrometer was entirely satisfactory in technical details and for use at ordinary temperatures, but the results obtained with it at temperatures below 0° C. were erratic. After a number of unsuccessful attempts to overcome the eccentricities, it was finally concluded that these were due to variable adsorption of moisture on the walls of the test chamber during sampling. Further development of the instrument must therefore be postponed until this difficulty can be overcome.

Dew-point Hygrometer

The two main difficulties encountered in the application of the dew-point principle to the determination of relative humidity at low temperatures are:

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[2] *Biophysicist, Food Storage and Transport Investigations.*

(i) The observed temperature must yield an accurate estimate of the true temperature of the surface on which dew is deposited, since the dew-point depression per unit change of humidity decreases with the air temperature. (ii) The small amount of moisture present in the air at low temperatures increases the time required for a visible deposit of dew to form, and unless the dew-point temperature is approached very gradually its observed value may be considerably below the true value.

Awbery and Griffiths (1) have described a dew-point instrument in which the first of these difficulties is largely overcome by pressing a thermocouple junction against the mirror on the reverse side to that on which dew is de-deposited. The rate of cooling of the mirror was only partially controlled, however, by regulating the rate of flow of paraffin oil, cooled with solid carbon dioxide, across the reverse side of the mirror. These authors observed a mean difference of 3% in relative humidity between the results obtained with the dew-point instrument and those obtained by the standard gravimetric absorption method.

In the instrument described in the present paper the difficulties mentioned above are both overcome, the first by having multiple thermocouple junctions contained in the mirror and connected in series to increase the thermoelectric potential developed, and the second by providing accurate temperature regulation of the cooling fluid so that the mirror may be cooled as slowly as desired. The accuracy attainable with the apparatus in its present form depends upon the rate at which the dew-point temperature is approached.

The principle of the apparatus can best be understood by reference to Fig. 1. The vessel C is filled to the indicated level with a non-freezing solution such as alcohol or ethylene glycol in water. The rest of the assembly, consisting of the liquid-circulating system, dew-point mirror, and thermocouples, is then put into place. In order to circulate the liquid, air is forced through the inlet tube A and passes out of the jet J below the liquid level. Air bubbles and liquid entrapped between them pass up through the tube B, across the under side of the mirror M to cool it, and down through the tube D, the liquid returning to the vessel C while the air escapes at E.

The mirror is made of copper, thinly plated with chromium, and has six holes drilled into its edge, each hole to accommodate a thermocouple junction T of 40-gauge copper and constantan wires. When in position, the junctions lie about 1 mm. beneath the surface of the mirror, and are held fast and insulated from the apparatus by a thin film of shellac. The mirror can be conveniently mounted by drilling two longitudinal holes in a brass rod, and recessing a portion of this rod as shown in the diagram. It has been found most convenient to immerse the second set of thermocouple junctions in a non-freezing solution in a Dewar vessel, and obtain their temperature with an accurate thermometer. The two sets of junctions are connected in series with a galvanometer calibrated to read directly the temperature difference between them.

The assembly shown in the diagram is immersed slightly beyond the liquid level in *C* in a non-freezing liquid bath provided with a small electric heater, and surrounded by a cooling medium. In practice, an ice formed by freezing a 23% solution of sodium chloride (F.P. −21° C.) has been found satisfactory for air temperatures down to −15° C. Solid carbon dioxide may also be used. With either of these substances a small air space separating the liquid bath from the cooling medium to retard the rate of cooling is an advantage. Cooling by mechanical means has not been attempted but should also be satisfactory. Accurate control of the cooling rate is obtained by manual control of the electric heater in the bath.

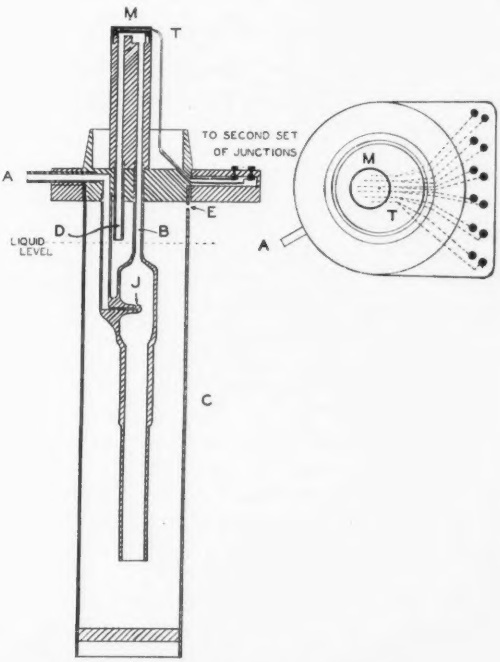

FIG. 1. *Diagram of dew-point hygrometer.*

An air stream of about two bubbles per second from a jet 0.045 in. in diameter is sufficient for satisfactory circulation of the liquid through the mirror. A small compressor with a safety valve and stopcock in the line to provide control has been found convenient, but a variety of other methods could doubtless be used to obtain the necessary slow stream of air. Displacement of air from a bottle by a liquid under a small head pressure, compressed air, or even a mechanically operated syringe bulb should serve the purpose.

Tests with a manually controlled arrangement of the apparatus at temperatures down to $-15°$ C., and over a range of 60 to 95% relative humidity showed that a precision of $\pm 0.5\%$ relative humidity was readily attained when the dew-point was approached at the rate of about 2° C. per hour. Although precision of this order is quite adequate for most practical purposes, it could doubtless be further increased by approaching the dew-point more slowly. There is no reason to believe that the apparatus would not work equally well at considerably lower temperatures. By providing photoelectric detection of dew formation it should be possible to incorporate the principle into an automatically operating and recording instrument. The development of such an instrument has been undertaken.

Acknowledgments

Grateful acknowledgment is made to Mr. E. A. Rooke for technical assistance in constructing the apparatus and making tests with it, and to Dr. W. H. Cook for valuable suggestions in its design.

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THE BIOLOGY OF THE MEADOW NEMATODE *PRATYLENCHUS PRATENSIS* (DE MAN) FILIPJEV 1936[1]

By R. J. Hastings[2]

Abstract

The meadow nematode completes its life cycle in 54 to 65 days—25 to 31 days from the larval stage to the adult, and 29 to 34 days from maturation to the second generation. Eggs are deposited by a single female at the rate of not more than one a day. The largest number of eggs laid by a single female in one place was sixteen, owing apparently to migratory habits. The total number of eggs from a single female could not be determined.

The adult male and female and all larval stages of this species are capable of entering the roots of oats. They are very susceptible to desiccation. No living nematodes were recovered from invaded root tissue that was allowed to dry. In moist excised oat roots, the nematodes remained viable for more than 30 days, but in water the majority died within the same period. A ten-minute immersion of infested oat roots in hot water will destroy the meadow nematode only when the temperature is 120° F., or higher.

Introduction

Recent investigations by Hastings and Bosher (1) have shown that the meadow nematode, *Pratylenchus pratensis* (de Man) Filipjev 1936, is capable of causing injury to many economically important plants, sometimes reducing growth rates by 50 to 75%. Hence the meadow nematode has to be accepted as an important plant parasite.

Goodey (2) has referred to the life history of the meadow nematode as similar to that of the burrowing nematode, *Rotylenchus similis* (Cobb) Filipjev 1936. He states that the infective stage is probably the first stage larva and that a period of four to five weeks elapses between maturation and the appearance of the second generation.

Experimental evidence was obtained by Hastings and Bosher that the first larval stage is an infective stage, but in addition the evidence proved that the other stages, including adult males and females, can enter oat roots. Both adults and larvae were found in one-day-old oat roots.

Experimental

Before a study of the life cycle of the meadow nematode was begun it seemed necessary to determine the range in size of each of the several larval stages and adults, and also to determine the ability of each of the stages in the life cycle to enter roots, in order to follow their development within root tissue.

Sizes of Adults and Larvae and Their Distribution in a Population from Oat Roots

Measurements of the length of 223 nematodes were made from a population in water that was obtained by suspending in water a quantity of infested oat roots. The data are presented in Table I.

[1] *Contribution No. 572, Botany and Plant Pathology, Science Service, Department of Agriculture, Ottawa, Canada. (Continuing the Series of the former Division of Botany.)*

[2] *Graduate Assistant, Grade I, Dominion Laboratory of Plant Pathology, Saanichton, British Columbia, Canada.*

TABLE I

THE SIZES OF ADULTS AND LARVAE OF THE MEADOW NEMATODE AND THEIR DISTRIBUTION IN A POPULATION FROM OAT ROOTS

Class	Number	Percentage of total	Length in mm.		
			Minimum	Mean	Maximum
1st stage larvae	74	33.2	.156	.187	.215
2nd stage larvae	37	16.6	.224	.258	.272
3rd stage larvae	17	7.6	.288	.326	.345
Pre-adult larvae	16	7.1	.345	.360	.388
Adult, male	34	15.2	.316	.360	.417
Adult, female	45	20.1	.360	.417	.489

Entrance of Adults and Larvae into Oat Roots

The entrance of distinct stages into oat roots was studied as follows: With the aid of a binocular microscope and capillary pipette, 50 to 75 specimens of each class of the meadow nematode were collected into separate Petri dishes. These suspensions were mixed with powdered peat and in each dish six oat seeds were sown. One week after the seeds germinated, the seedlings were removed and prepared for microscopic examination for the presence of nematodes by washing the roots in running water and clearing and staining them with lacto-phenol and acid fuchsin. All stages were found in the roots.

LIFE CYCLE OF THE MEADOW NEMATODE

The life cycle of the meadow nematode was studied by following the development of the first stage larvae to maturity in oat roots in relation to time, and by determining the time between entrance of adults into oat roots and the earliest appearance of larvae of the second generation.

A. Development of the First Stage Larvae to Adults

About 100 first stage larvae were collected in each of three Petri dishes and mixed with powdered peat. Oats were sown in these cultures and the nematodes entered the roots soon after they germinated. The seedlings were kept in the medium for five to six days, and were then transferred to autoclaved soil in individual 4-inch pots. One or more plants were examined at intervals by the lacto-phenol technique. The examinations revealed the presence of adults 25 to 31 days after the larvae entered the roots. The difference between maximum and minimum values is the period that the plants were exposed to infection in the Petri dishes. (Table II.)

B. Time Between Entrance of Adults into Oat Roots and Appearance of Second Generation Larvae

The method in this experiment was similar to the last, except that adult females and a few males were used as inoculum. Three experiments were conducted, and the results are presented in Table III. In the first experiment, examinations were made more frequently in order to obtain if possible

the rate of egg deposition, the egg output and the egg incubation period. The data from the first experiment revealed that a female residing in the roots for 12 to 17 days laid 12 eggs. No eggs had hatched, hence the egg incubation period is more than 12 days. The data in the second experiment disclosed that 28 to 32 days after the adults entered the roots there were 16-egg nests and only slight evidence of hatching, as 37 females, 235 eggs and only 9 larvae were counted. Most of the 16-egg nests contained no

TABLE II

THE DEVELOPMENT OF FIRST STAGE LARVAE TO ADULTS IN RELATION TO TIME

Date	Days nematodes were in roots		Nematodes found in roots					
	Minimum	Maximum	1st stage larvae	2nd stage larvae	3rd stage larvae	Pre-adult larvae	Adults	Eggs
Feb. 14	4	10	2	4	0	0	0	0
Feb. 25	15	21	0	1	1	0	0	0
Feb. 28	18	24	2	5	5	2	0	0
Mar. 7	25	31	0	1	3	4	2	0
Mar. 11	29	35	0	0	2	2	3	0
Mar. 28	46	52	0	0	0	0	5	2
Apr. 4	53	59	0	0	0	0	1	2

larvae, hence the egg incubation period is more than 16 days. The data in the third experiment showed that 26 to 30 days after the adults entered the roots three females had produced 30 eggs, of which one had hatched. After 33 to 37 days, however, 25 females, 254 eggs, and 56 larvae were counted from the roots examined. In several of the nests there were as many as four larvae, probably four days old. If four days are deducted from 33 to 37 days, the minimum time between entrance of adults and the appearance of larvae would be 29 to 34 days. The life cycle of the meadow nematode is thus 54 to 65 days, 25 to 31 days for the first stage larvae to reach adult stage, and 29 to 34 days from maturation to the appearance of a second generation. The life cycle of *P. pratensis* is apparently somewhat similar to *Ditylenchus radicicola*, the root gall nematode, which was recently studied by Goodey. He reported that the life cycle took 56 to 64 days (3).

The data contained in Table III also showed that the maximum egg output of any single female at any time throughout the duration of the experiments was always less than the age of the plants in days, which suggests that the female nematode does not lay more than one egg a day.

Observations Concerning Some of the Habits of the Meadow Nematode

The nematodes that entered oat roots from peat cultures were invariably found in the older parts of the tissue, seldom in or near the root tip. The larvae were headed generally in the direction of the root tip. The adults

were headed in all directions. The nematodes are vagrant but do not seem to travel very far in the root tissue. The course travelled by females could be followed by the position of the eggs. The eggs are usually grouped together, but occasionally they may be spread out. In this case the most distant egg was not more than 2 mm. from the parent. After depositing 15 to 16 eggs, the female usually migrates. Migration occurs via the soil. In peat cultures the nematodes have travelled through $3\frac{1}{2}$ in. of medium in three days. In soil the migration rate may be as rapid. Owing to this habit of migration, the number of eggs that a single female produces could

TABLE III

TIME BETWEEN ENTRANCE OF ADULTS INTO OAT ROOTS AND APPEARANCE OF EGGS AND SECOND GENERATION LARVAE

Date of examination	Days in roots		Nematodes found in the roots			Number of eggs laid by individual females		
	Min.	Max.	Adults	Eggs	1st stage larvae	Min.	Max.	Average
Series 1								
Jan. 14	0	3	3	2	0	0	2	0.5
Jan. 18	2	7	7	7	0	0	4	1.0
Jan. 21	5	10	8	15	0	0	4	1.8
Jan. 24	8	13	16	81	0	2	9	5.0
Jan. 26	10	15	4	27	0	5	9	6.7
Jan. 28	12	17	4	44	0	9	12	11.0
Series 2								
Feb. 7	0	4	3	0	0	0	0	0
Feb. 9	2	6	102	21	0	0	2	0.2
Feb. 14	7	11	3	2	0	0	2	0.6
Feb. 18	11	15	4	12	0	0	6	3.0
Feb. 21	14	18	3	10	0	0	6	3.3
Feb. 28	21	25	18	117	0	0	12	6.5
Mar. 7	28	32	37	235	9	0	16	7.0
Mar. 11	32	36	7	56	7	0	14	9.0
Series 3								
Feb. 18	9	13	4	12	0	2	6	3.0
Feb. 24	15	19	48	341	0	3	9	7.0
Feb. 28	19	23	1	15	0	15	15	15.0
Mar. 3	22	26	12	126	2	5	15	10.5
Mar. 7	26	30	3	29	1	0	16	10.0
Mar. 14	33	37	25	254	56	0	16	12.0

not be determined. The largest number in a nest, traceable to an individual female, was 16, but reference to Table III shows that at the end of each series of tests there were females with no eggs close to them, indicating that they had migrated from some other position. Observations of a 26-day-old plant definitely substantiate this. The plant had three roots. Root 1 contained no nematodes, but four nests of 7, 13, 13, and 12 eggs respectively. Root 2 had two females with no eggs and another female with nine eggs. Root 3 contained six females but only five nests of eggs.

The eggs in oat roots are usually found in the cortex, generally in an axial position to the root, but eggs in a transverse position were also found, in one instance 3 in a nest of 14. This observation differs from that of Steiner with respect to the meadow nematode in rice roots (4). Steiner reported finding no eggs in a transverse position and suggested that there may be a possible connection between the regular axial position of the eggs and either (a) the mode of deposition or (b) the character of root cells and type of tissue.

Effect of Desiccation on the Meadow Nematode

Desiccation proved fatal to the meadow nematode. In one experiment, infested oat roots were air-dried for three days and were then suspended in water; but although there were numerous nematodes in the tissue, none emerged, as all had died. In another experiment, freshly excised infested oat roots were placed in water. In 48 hr. there was an abundance of nematodes in the suspension. The water was allowed to evaporate, and the dish was kept dry for 24 hr., then water was added. None of the nematodes regained their motility; they had died from the effects of desiccation.

Survival in Excised Oat Roots and in Water

In excised oat roots that were maintained in water, the meadow nematode survived for a period of four weeks. Infested oat roots were transferred every few days to a different dish containing water, and in each of seven suspensions there was an emergence of the nematodes into the water. On the thirtieth days of the experiment, an examination was made of the nematodes in each suspension, and the number and percentage of living and dead nematodes was determined.

TABLE IV

Survival of the meadow nematode in excised oat roots and in water

Date of suspension of excised infested oat roots	Period nematodes were free-living in water (days)	Number of nematodes in suspension	Number alive	Percentage alive
1st – 6th	24 – 30	112	19	16
6th – 10th	20 – 24	37	18	48
10th – 14th	16 – 20	87	47	53
14th – 18th	12 – 16	28	18	64
18th – 22nd	8 – 12	10	10	100
22nd – 26th	4 – 8	6	6	100
26th – 30th	1 – 4	3	3	100

In the case of those emerging early from the oat roots, four weeks in water usually exhausted the nematodes and caused the death of over 80%. However, even after six weeks there were still a few survivals. The nematodes that remained quiescent within the root and conserved their energy were able to survive longer than those in the free-living condition. The stage of development was not a factor in these survivals.

Minimum Lethal Temperature of the Meadow Nematode

A 10-minute immersion of infested oat roots in water at 120° F. destroyed the meadow nematode. Immersions of infested roots at lower temperatures failed to kill the nematodes, but delayed their emergence from the roots.

Oat plants were immersed in hot water for 10 min. and the excised roots were afterwards suspended in water for the emergence of surviving nematodes. Observations made on the seventh day of suspension disclosed emergence of survivors from roots immersed at 100° F. and 105° F. but not from roots immersed at higher temperatures. On the tenth day, there was emergence from roots treated at 110° F., and on the fourteenth day, from roots treated at 115° F., but up to the thirtieth day, no nematodes came out of the roots treated at 120° F. Thompson (5) has reported that lily of the valley crowns have been freed from the meadow nematode by immersing the plants for 30 min. at 113° F. In the present study, infested oat roots were immersed at the same temperature for the same period but nematode survival was found.

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A STUDY OF BACTERIA CONTAMINATING SIDES FOR WILTSHIRE BACON WITH SPECIAL CONSIDERATION OF THEIR BEHAVIOUR IN CONCENTRATED SALT SOLUTIONS[1]

By E. H. Garrard[2] and A. G. Lochhead[3]

Abstract

Forty microbial types, in which micrococci predominated, were found as representative of pre-curing contamination, including strains resembling organisms found in bacon slime.

Varying degrees of salt tolerance were noted, micrococci showing the greatest ability to grow at higher concentrations. With 25% sodium chloride only two species showed growth. Tests with species reducing nitrate to nitrite at 5% salt concentration showed that as the salt concentration increases, nitrate reduction occurs with a progressively smaller proportion of those showing growth. This suggests that nitrate reduction in curing pickle is a function of the true halophiles rather than of the pre-curing contaminants.

The organisms could be placed in five groups depending upon their salt resistance (ability to survive) in salt solutions and in curing pickle. Much greater resistance to salt was displayed in curing pickle than in salt broths of similar sodium chloride content. Pickle appeared to possess substances tending to neutralize the toxic effect of salt, the action being "protective" rather than "stimulative".

Many types of bacteria constituting original contamination are considered able to survive the pickling process. Although the findings do not point to any pronounced activity of these in pickle, their ability to survive opens the possibility of their becoming active after pickling and contributing to storage defects. The results justify the adoption of measures for the utmost plant sanitation in Wiltshire processing.

Introduction

The greater part of the bacon exported from Canada is in the form of Wiltshire sides, which are pickled, drained, and baled in Canada and receive further treatment in England. From time to time, sides when received are found to show off-flavour, discoloured areas, or slime, the latter principally on the membrane of the rib tissue. Bacteriological examination of slime may show considerable numbers of micro-organisms, many of which are non-halophilic in nature, despite the fact that the sides have been cured in strong brine and, after draining, have a salt content of some 4 to 5%. This suggests that undesirable bacteria either may be present in the pickle or may contaminate the sides during draining, wiping, and baling operations. The extent of the defects on the sides will depend naturally on their development as affected by temperature, humidity, and time of storage before arrival of the sides on the market.

It is well known that carcasses may be contaminated during operations on the killing floor of slaughterhouses and packing plants and that this contamination may be augmented by subsequent handling of the meat. It has

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been shown by Haines (2) that the initial bacterial load noticeably affects the storage life of the meat. Obviously the storage life will also be dependent upon the extent to which contaminating bacteria have been allowed to develop. The importance of such factors as temperature, relative humidity, and carbon dioxide concentration during storage has been shown by a number of workers, such as Schwartz and Schmid (5), Haines (1), and Scott (6, 7, 8). For the fresh and chilled meat trade, the prevention and control of microbial contamination are thus matters of the greatest concern, and standards of cleanliness are advised to keep contamination by, and growth of, micro-organisms at a minimum.

In Wiltshire processing, involving the curing of sides in concentrated brine under conditions in which bacteria are considered essential agents, the significance of the initial contamination is by no means clear. In our packing plants, hygienic measures are adopted to minimize contamination prior to curing, on the justifiable assumption that micro-organisms contaminating meat, if allowed time and suitable temperature, may cause various types of spoilage. However, there is little or no exact knowledge of the bactericidal or bacteriostatic effect of the high salt content of the curing pickle on contaminating organisms, nor on the more practical questions as to the possible role of such contaminants in the cure, or the ability of certain types to survive the pickling and act as possible agents of spoilage during the subsequent storage period.

Preliminary studies (4) in the analysis of curing pickle have shown the presence in the brine of appreciable numbers of non-halophilic organisms, able to resist, if not multiply in, high concentrations of salt. Observations (unpublished) on the surface load of Wiltshire sides at different stages of the processing showed that organisms able to develop without salt were an important group of bacteria prior to pickling, and likewise comprised a group that definitely increased during the storage of the pickled sides after baling. Taken together, the above sets of findings suggest that deleterious organisms that may contaminate sides prior to pickling, may carry through and cause defects on cured, stored sides. The aim of the present study was to obtain information of a more direct nature on this point.

Experimental

The main purpose of the experiments here reported was to study the types of bacteria contaminating sides of Wiltshire bacon prior to curing, and to note particularly their ability to tolerate various concentrations of salt, with distinction between capacity for growth and for survival. Samples were taken from sides just prior to pumping and pickling, as being representative of plant contamination preceding the cure. To afford further information and obtain representative types of organisms from various sources of contamination, samples were also taken from freshly slaughtered hogs, from sides in the chill room just before removal to the cutting room (approx. 40 to 48 hr. after killing), and from sawdust, air, and wall scrapings in the chill room.

Surface contamination of the sides was studied by a filter paper impression method, using Whatman No. 3 filter paper cut into squares of 4 sq. cm. and sterilized in Petri dishes in hot air at 120° C. Samples were taken by pressing a square of paper firmly against the meat tissue for 20 sec. with sterile forceps, and then dropping it into a 750-ml. Erlenmeyer flask containing 500 ml. physiological salt solution and 75 gm. broken glass. Various methods of estimating surface contamination of meat have been used. The impression method was selected in preference to those involving scraping, or cutting out measured areas, as it obviated any mutilation of the tissue. From each side, impressions were taken as follows: two from lean flesh, two from centre rib section and one from lower short ribs. To each flask were added 20 squares representing impressions from four sides. Hogs were grouped into lots of four, picked at random as they came from the killing and dressing floor on their way to the cool room. From each group (eight sides) two flasks were prepared. In all, 32 sides were used for sampling, marked so they could be followed to the cutting room.

At the laboratory, flasks were shaken for 10 min. until the filter paper was disintegrated, and the contents of each pair of flasks were mixed in a previously sterilized 2000-ml. flask. From the combined suspension of 40 squares, further dilutions were prepared with physiological saline blanks, and plates were poured using nutrient agar and agar media containing respectively 5, 10, and 15% sodium chloride*. All plates were incubated at room temperature (approx. 20° C.). Nutrient agar plates were counted after five days, 5% and 10% salt plates after two weeks, and 15% salt plates after three weeks. Similar media were used for the quantitative determination of organisms in sawdust, air, and wall scrapings of definite areas.

Pre-curing Contamination

Though the main object of the work was to obtain representative types of bacteria for detailed study rather than a systematic quantitative examination of plant contamination, quantitative data illustrating the trend of surface contamination at different times are shown in Table I. In Table II is found a summary of results from the examination of sawdust, wall scrapings, and air.

As expected from an examination of this kind, in which the extent of contamination is varied and accidental direct contact with walls, clothing, etc., may make for great localized differences, variations are noted. The method of sampling involves additional errors due to the possibility of touching the same area twice and of covering more grossly contaminated spots from which contamination may be later reduced in various trimming, cutting, and wiping processes. The results show, however, that sides for Wiltshire bacon may become definitely contaminated with bacteria prior to pickling, within the space of some 40 to 48 hr.

* *In this paper, % sodium chloride = grams per 100 ml. of solution.*

The findings suggest that under conditions in which sides are removed, promptly after killing, to a chill room of approximately 32° F., the microbial load at the end of cutting is the result of direct contamination rather than growth. It is possible for sides to leave the chill room with little or no increased microbial load; when increased counts are noted, they are regarded as due largely to direct contact infection. Table II suggests that air is normally a relatively insignificant source of contamination, whereas the numbers of bacteria present in used sawdust or in wall scrapings point to the danger of greatly increased contamination of the meat resulting from accidental contact with such sources. The increased counts after cutting are due to the handling in the cutting room involving various cutting, sawing, trimming, and handling operations carried out during a period of approximately one-half hour.

TABLE I

SURFACE CONTAMINATION OF WILTSHIRE SIDES PRIOR TO CURING

Group No.	—	Count per sq. cm. on agar			
		0% NaCl	5% NaCl	10% NaCl	15% NaCl
1	Freshly killed	87	87	19	6
	After cooling	1,110	1,090	450	220
	After cutting	3,890	4,860	2,260	790
2	Freshly killed	3,550	5,350	3,031	750
	After cooling	31,500	40,560	5,150	100
	After cutting	3,750	2,030	1,820	340
3	Freshly killed	630	220	100	50
	After cooling	660	150	50	20
	After cutting	12,130	7,440	1,220	490
4	Freshly killed	150	190	170	40
	After cooling	250	130	70	6
	After cutting	10,810	7,810	2,140	840

TABLE II

BACTERIAL COUNTS—VARIOUS SOURCES OF CONTAMINATION

Source	Agar plate count			
	0% NaCl	5% NaCl	10% NaCl	15% NaCl
Fresh sawdust, per gram	7,000	9,000	1,000	1,000
Used sawdust, per gram	216,000,000	190,000,000	73,000,000	750,000
Bloody sawdust, per gram	14,400,000,000	24,640,000,000	8,800,000,000	700,000
Wall scrapings, per gram	67,500,000	47,500,000	46,200,000	38,700,000
Exposed plates[1]—floor	180	125	15	5
Exposed plates—raised	90	33	23	15

[1] *Area, 60 sq. cm.; exposed to air 15 min.*

Classification of Organisms

Examination of the plates of nutrient agar and of salt agar showed variations in the bacterial types developing. Special attention was given the "after cutting" series in an estimation of the relative incidence of different types. From plates or sectors of plates all colonies were picked and transferred to agar slants for further study. The percentage distribution of the various morphological types is illustrated in Fig. 1, which shows the changing proportions of the groups as they vary with the salt content of the medium.

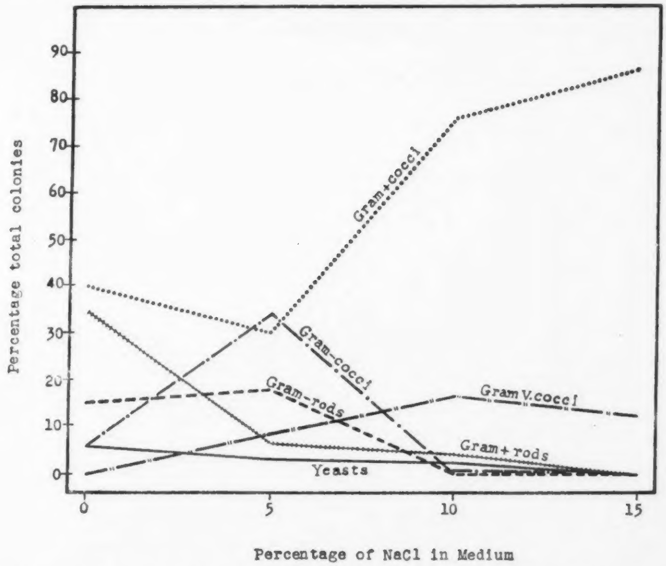

FIG. 1. *Distribution of various morphological types on plates of different salt concentration, representing contamination of sides after cutting.*

For more detailed study of the hundreds of colonies isolated it was necessary to concentrate on one isolation group. It was felt that the organisms on 5% salt agar plates would be most representative of those comprising initial contamination of meat to be pickled. This medium showed a well-assorted bacterial flora, with all morphological groups well represented, and gave comparatively high total counts. Facultative halophiles, which might be important in pickle, should be well represented on 5% salt agar, to the exclusion of certain non-halophiles. Finally, as 5% salt is a favourable concentration for isolating types occurring in bacon slime, it was felt that this concentration would be most likely to include organisms able to cause spoilage. Accordingly, 100 cultures from the 5% salt agar plates of the "after cutting" series, together with a group of 185 cultures from plates of the same medium prepared from sawdust, air, wall scrapings, and freshly slaughtered hogs,

were studied microscopically and culturally with the object of eliminating similar strains and reducing the number to those that could be regarded as different type species. For this differentiation, reliance was placed on Gram staining, nitrate reduction, gelatin liquefaction, lipolysis, litmus milk reaction, and fermentation of dextrose. All test media contained 5% sodium chloride.

The relative abundance of the morphological groups represented in the 285 cultures isolated from 5% salt agar plates is shown in Fig. 2, organisms from the "after cutting" series being compared with those from various sources before cutting. Micrococci, Gram positive and Gram negative, were found to comprise the most abundant groups, followed by Gram negative rods,

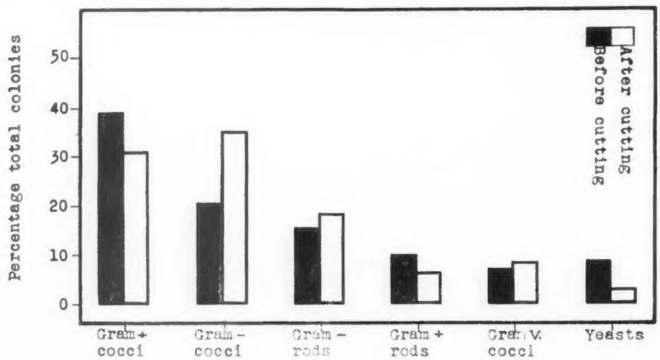

FIG. 2. *Relative incidence of different groups of organisms from various sources before cutting, and from the "after cutting" series.*

many of which were highly pleomorphic forms. A numerically important group, the classification of which presented some difficulty, was included as Gram negative cocci. These organisms were characteristic oval forms which might also be regarded as coccoid rods. As a group these showed relatively pronounced lipolytic action and were similar to organisms found by Landerkin (3) to be the most abundant types appearing on 5% salt agar plates from certain samples of slime on cured Wiltshire sides.

As a result of the comparative study of 100 cultures from the "after cutting" series, 28 bacterial cultures and one yeast were regarded as different types. Examination of the group of 185 cultures revealed 11 additional bacterial types. Forty cultures therefore were regarded as types representative of the 285 original isolations. Further study was made of the cultural characteristics of these organisms, details of which need not be given here. However, a summary of the 39 bacterial types representing pre-curing contamination is given in Table III, the organisms being grouped on the basis of morphology and more important physiological properties.

TABLE III

SUMMARY OF BACTERIAL TYPES REPRESENTING CONTAMINATION PRIOR TO CURE

—	No. of different types	Chromogenic	Motile	Spores	Gelatin liquefaction	Nitrate reduction	Lipolysis	H_2S production	Acid in		
									Dextrose	Sucrose	Lactose
Micrococci, Gram pos.	10	5	0	0	5	6	6	4	8	7	4
Micrococci, Gram neg.	6	0	0	0	0	4	5	2	0	0	0
Micrococci, Gram. var.	4	0	0	0	1	2	1	1	4	4	2
Rods, Gram. pos.	9	2	0	1	4	4	1	1	2	2	0
Rods, Gram neg.	10	2	4	0	3	6	2	3	3	2	0
Total	39	9	4	1	13	22	15	11	17	15	6

Relation of Organisms to Salt

The effect of salt on micro-organisms has been given much attention and there has accumulated, in consequence, a voluminous literature which need not be reviewed here. It is known that organisms from a salt-free environment can tolerate salt to varying degrees, with evidence of some stimulation by lower, and eventual suppression by higher concentrations. The degree of tolerance to salt is further modified by other environmental factors such as temperature, aeration, pH, organic nutrients, amount of inoculum, age of culture, and microbial association. It is further apparent that certain types, such as micrococci, from salt-free sources are able to withstand higher salt concentrations than others. From salt environment, on the other hand, both from natural and "industrial" sources, organisms have been found that prefer or require various concentrations of salt, some thriving best in an environment approaching saturation. Among such organisms micrococci are less predominant. That a considerable degree of adjustment to salt may occur seems well established, though there is still difference of opinion as to the suitability of, and distinction between, such terms as "obligate halophile", "facultative halophile", "salt tolerant", and "salt resistant".

Since organisms contaminating Wiltshire sides prior to curing are subjected to the influence of a salt pickle approximating saturation, it was considered a matter of practical interest to study the relation of the microbial types found, in various concentrations of sodium chloride. Information on this point should permit a better estimate of the importance of pre-curing contamination and its possible relation to later defects of the cured product. Tests were therefore made of the "salt tolerance" and "salt resistance"* of 40 microbial types representing contamination, to which were added, for comparison, seven other organisms from various sources.

* *For the purpose of this paper, "salt tolerance" refers to ability to grow, and "salt resistance" ability to remain viable, in an environment of definite salt concentration.*

Methods

Salt tolerance. From eight-day cultures of the organisms on 5% salt agar, faintly turbid suspensions were made in 5% sodium chloride solution, and one loopful of the suspension was used for inoculation. Heavy inocula were avoided, in view of the findings of certain workers (9, 10), who showed that mass effect caused by the addition of large inocula may increase salt tolerance, and thus tend to neutralize any inhibitive effects. Transfers were made to a series of slants of nutrient agar containing respectively 0, 5, 10, 15, 20, and 25% sodium chloride, in addition to 0.1% potassium nitrate. Transfers were also made by loop stab to a similar series of semi-solid media of the same composition except that 0.08% agar was used. Although the amount of agar used was so small that the medium did not appear to differ from straight broth, tests showed that when growth occurred it appeared as a suspended localized growth, which showed to advantage compared to a general distribution in broth, and made the recognition of bacterial multiplication easier.

Inoculated tubes were incubated at room temperature for two weeks in the case of 0 and 5% sodium chloride media, and for three weeks with the higher concentrations. Tightly rolled plugs allowed but a minimum of evaporation. The tubes were examined for growth and tested for nitrites. Organisms showing growth were adjudged to have tolerated the salt concentration in question.

Salt resistance. By means of a measured loop, equivalent amounts of 10-day cultures were transferred to 10 ml. of 5% sodium chloride solution. This was agitated and an endeavour made to establish as uniform turbidity as possible. One-tenth ml. of suspension of each culture was transferred to two tubes of 5% sodium chloride broth, to one tube each of 10, 20, and 30% sodium chloride broth and to one tube of sterile pickle (29.75% sodium chloride). The salt broths contained, in addition to sodium chloride, 0.1% potassium nitrate, 0.15% beef extract, and 0.25% peptone. The pickle was a filtered curing brine, as used for Wiltshire sides. All tubes contained 10-ml. quantities.

From one of the duplicate 5% sodium chloride tubes, plates were poured with 5% salt agar and incubated at room temperature for two weeks. The counts gave the original count per ml. at time of inoculation. At the end of 5 and 10 days' incubation at room temperature, times that correspond approximately with the duration of cure at various plants, estimates were made of the numbers of organisms in each inoculated tube by plating on 5% salt agar. Since larger amounts taken from the tubes of the higher salt concentration would affect the sodium chloride concentration and hence the counts of the agar plates, transfers were made by loop. Calibration for each sodium chloride broth was necessary owing to the effect of specific gravity on the volumes delivered by the same loop. Preliminary tests were made in weighing loopfuls of each salt concentration. Repeated weighings showed that out of 120 loopfuls there was rarely more than one or two loopfuls difference each

time. In plating, the loopful was mixed with sterile 5% salt solution in the plate and gently tapped to rid the loop of all its contents. This followed analogous procedure in standardizing. Dilution, if necessary before plating, was made in sodium chloride solution. Plates were incubated at room temperature for two weeks before counting.

Results

Results from the salt tolerance tests showed good agreement between the agar and semi-solid inoculations. The semi-solid medium showed if anything slightly better growth, and in Table IV are summarized the results from this medium, arranged for the morphological groups. Gram negative micrococci show a pronounced decline in salt tolerance between sodium chloride concentrations of 10 and 15%, whereas Gram positive micrococci display a greater salt tolerance. There was little difference between the Gram positive and Gram negative rods, considered as groups. The tests show that many organisms isolated on 5% salt agar from an apparently salt-free environment

TABLE IV

SALT TOLERANCE (CAPACITY FOR GROWTH) OF MICRO-ORGANISMS REPRESENTING CONTAMINATION PRIOR TO PICKLING

Morphological group	No. of types in group	Number showing growth in semi-solid medium					
		0% NaCl	5% NaCl	10% NaCl	15% NaCl	20% NaCl	25% NaCl
Contaminants from plant							
Micrococci, Gram pos.	10	10	10	10	9	3	1
Micrococci, Gram neg.	6	6	6	6	1	1	0
Micrococci, Gram. var.	4	4	4	3	3	0	0
Rods, Gram pos.	9	9	9	9	5	2	1
Rods, Gram neg.	10	9	10	9	5	2	0
Yeasts	1	1	1	1	0	0	0
Total	40	39	40	38	23	8	2
Control Cultures							
Staph. aureus		+	+	+	+	−	−
Esch. coli		+	+	−	−	−	−
Achromobacter sp. from slime (No. 44)		+	+	+	+	+	−
Micrococcus sp. from pickle (No. 42)		−	+	+	+	+	−
Achromobacter sp. from pickle (No. 45)		−	−	+	+	+	+
Micrococcus sp. (No. 46)		+	+	+	+	+	−
Micrococcus sp. (No. 47)		+	+	+	+	+	−

in a packing plant can tolerate relatively high sodium chloride concentrations, more than one-half being able to grow on media ranging from 0 to 15% sodium chloride, and one-fifth up to 20%. A concentration of 25% sodium chloride, however, appears to be definitely inhibitive to organisms isolated and carried on a 5% sodium chloride medium. That some adjustment to high salt environment of pickle is possible, however, is suggested by the range of adaptability shown by many types.

Since part of the value of a pickle flora depends upon the presence of nitrate-reducing bacteria, cultures were tested for nitrite. Of those shown in Table IV, 26 species reduced nitrate to nitrite in 5% sodium chloride medium. In addition to the original series with light inoculum, a special series was prepared in which heavy inoculations were made from the 26 nitrate-reducing organisms. Results are shown in Table V. It is of interest to note from the

TABLE V

COMPARISON OF GROWTH AND NITRATE REDUCTION AT DIFFERENT SALT CONCENTRATIONS

26 species reducing NO_3 at 5% NaCl concentration	NaCl concentration					
	0%	5%	10%	15%	20%	25%
Light inoculum						
Growth	24	26	26	15	8	3
Nitrate reduction	24	26	24	11	4	1
Heavy inoculum[1]						
Nitrate reduction	24	26	24	12	6	1

[1] *Owing to heavy inoculum, growth could not be determined in this series.*

"light inoculum" series, that growth up to a concentration of 5% sodium chloride was accompanied by nitrate reduction. As the sodium chloride concentration of the medium increased, however, nitrate reduction occurred with a progressively smaller proportion of the species showing growth, until at 25% sodium chloride concentration the only organism showing reduction was one of the "controls", a halophile isolated from curing pickle. Mass inoculation produced relatively little effect on nitrate reduction. The findings are of interest in showing that nitrate reduction is interfered with before growth is suppressed, and suggest that reduction of nitrate in curing pickle is a function to be ascribed to the true halophiles rather than to the pre-curing contaminants.

In the salt resistance experiments, in which 47 cultures were placed in five salt environments and initial, 5-day, and 10-day (and in one set, 30-day) counts recorded, 564 analyses were made. In portraying this mass of data, ranging from very high to very low counts, it was felt that the findings could best be summarized in, (a) a graph showing the trend of groups according to their behaviour in salt, and (b) a table expressing the reaction according to morphological types. These are shown respectively in Fig. 3 and Table VI.

According to the action of the organisms in salt solution, five distinct trends were shown by the counts (Fig. 3).

Groups 1 and 2. Closely related groups, comprising nine species of Gram positive micrococci, four Gram positive and two Gram negative rods, which maintained their numbers relatively well in 20% and 30% sodium chloride and in pickle. Group 2 differed from Group 1 by showing higher counts in pickle than at the start, the only group to show this.

TABLE VI

SALT RESISTANCE (SURVIVAL) OF MICRO-ORGANISMS REPRESENTING CONTAMINATION PRIOR TO PICKLING

Morphological group	No. of types group in	Days	5% NaCl				10% NaCl				20% NaCl				30% NaCl				Pickle (29.75% NaCl)			
			Inc.	Stat.	Dec.	Inh.	Inc.	Stat.	Dec.	Inh.	Inc.	Stat.	Dec.	Inh.	Inc.	Stat.	Dec.	Inh.	Inc.	Stat.	Dec.	Inh.
Micrococci, Gram pos.	10	5	10				9		1			4	5	1		4	4	2		9	1	
		10	10				8		1	1		3	4	3		2	5	3		8	2	
Micrococci, Gram neg.	6	5	6				4	2				2	3	1			4	2	1	1	4	
		10	6				5		1			1	4	1			3	3		1	5	
Micrococci, Gram var.	4	5	4				4				1	1	1	1	1		1	2	1	2		1
		10	4				2	1		1	1		1	2			1	3	1	1	1	1
Rods, Gram pos.	9	5	9				6	1	2			3	6			2	3	4		5	4	
		10	9				5		4			3	4	2		2	2	5		4	5	
Rods, Gram neg.	10	5	10				5	1	2	2	1	2	4	3	1		5	4	1	3	3	3
		10	10				6		1	3		4	3	3		1	2	7	1		5	4
Yeast	1	5	1						1					1				1				1
		10				1				1				1				1				1
Total	40	5	40				28	4	6	2	2	12	19	7	2	6	17	15	3	20	12	5
		10	39			1	26	1	7	6	1	11	16	12		5	13	22	2	14	18	6
Staph. aureus		5	×				×					×					×				×	
		10	×				×						×				×				×	
Esch. coli		5	×				×						×				×				×	
		10	×							×				×				×				×
Achromobacter sp. (No. 44)		5	×				×					×					×			×		
		10	×				×						×				×			×		
Micrococcus sp. (No. 42)		5	×				×					×					×			×		
		10	×				×				×						×				×	
Micrococcus sp. (No. 46)		5	×				×						×			×				×		
		10	×				×						×			×				×		
Micrococcus sp. (No. 47)		5	×				×						×				×			×		
		10	×				×						×			×				×		

Inc. = *Increase over initial counts of 100% or more.* Dec. = *Decrease in count of 50% or more.* Stat. = *Within the above limits for increase or decrease.* Inh. = *Inhibition. No growth on plates.*

Group 3. Four Gram positive, three Gram negative, and one Gram variable species of micrococci, two Gram positive and one Gram negative rods, which declined in numbers from 5 to 30% sodium chloride solution, but maintained numbers up to 10 days in pickle, though declining after one month.

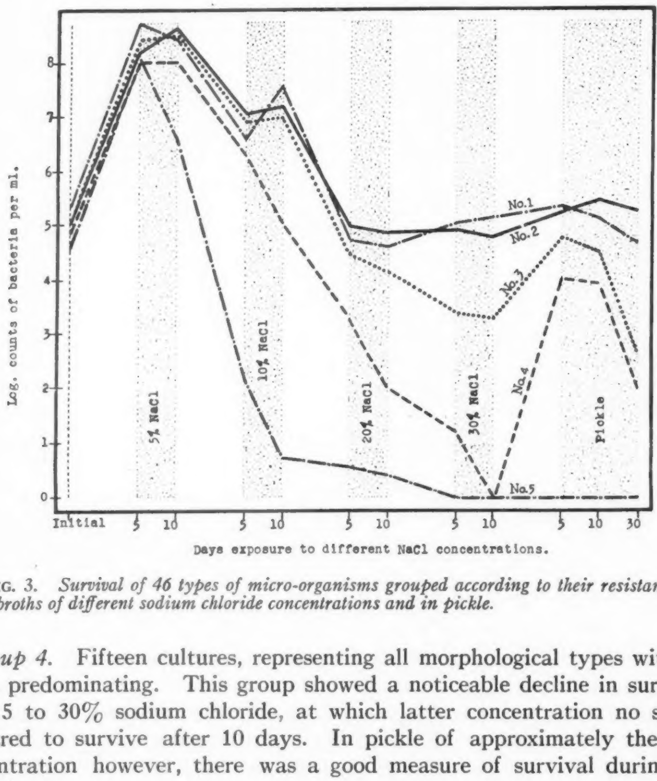

FIG. 3. *Survival of 46 types of micro-organisms grouped according to their resistance in salt broths of different sodium chloride concentrations and in pickle.*

Group 4. Fifteen cultures, representing all morphological types with rod forms predominating. This group showed a noticeable decline in survivors from 5 to 30% sodium chloride, at which latter concentration no species appeared to survive after 10 days. In pickle of approximately the same concentration however, there was a good measure of survival during the same time.

Group 5. Four Gram negative rods, together with the one yeast type. These organisms were the most susceptible, declining rapidly in sodium chloride concentrations above 5% and showing no evidence of survival in either 30% sodium chloride or in pickle.

Reactions according to morphological types are given in Table VI, and summarized according to the values "increase", "decrease," "static", or "inhibition", corresponding to standards* set on the basis of the plate counts made after 5 and 10 days respectively. From the table may be seen the relatively greater resistance of Gram positive micrococci and the comparative

* *For definition of terms see footnote to Table VI.*

susceptibility of Gram negative rods towards higher salt concentrations. The most striking finding is the much greater resistance displayed in pickle than in salt broths of similar sodium chloride content. Whereas 15 species were completely inhibited after 5 days, and 22 species after 10 days in 30% sodium chloride broth, but 5 and 6 species respectively were inhibited in pickle of almost identical sodium chloride concentration. On the other hand, whereas only 5 species showed static counts at the end of 10 days in 30% sodium chloride solution, 16 species showed static or increased counts in pickle, and these comprised 65% of the total number of organisms representing original contamination. It appears that curing pickle possesses substances that neutralize or mask the toxic or inhibitive action of high salt concentration, despite the fact that it was sterilized at 15 lb. for 30 min., filtered through paper to remove precipitate, and re-sterilized. Comparing the pickle totals with those for the sodium chloride broths, the inhibitive effect of the former is less than that of 20% sodium chloride broth, approximating that of a solution of one-half its actual salt strength. That the decreases and inhibitions in the salt broths were not owing to lack of nutrient is borne out by the high increases in the lower salt concentrations. The effect noted in pickle is "preservative" rather than "stimulative", as seen from the totals in the "static" column (Table VI). This is further supported by tests of plating media prepared for pickle, which showed no advantage over other media of similar sodium chloride strength.

To examine the possibility that organisms introduced to an environment of 30% sodium chloride might adjust themselves to better growth on higher concentration of salt agar than on the 5% sodium chloride medium used, all cultures that showed inhibition in 30% sodium chloride broth were subjected to a control test in which plating was made on 10 and 15% sodium chloride agar as well as on the regular 5% sodium chloride medium. In no case did cultures that showed inhibition, as judged by plating on 5% agar, show growth on the 10 or on the 15% sodium chloride agars, permitting the deduction that with the 23 species studied the action of 30% salt involved a toxic effect rather than an adjustment to higher salt concentrations.

It appears that many types of organisms, constituting original contamination of Wiltshire sides, are able to survive the pickling process. Although the findings do not point to any pronounced activity of such organisms in pickle, either beneficial or deleterious, their ability to survive opens the possibility of their becoming active on sides after pickling, and contributing to storage defects. The findings therefore point unmistakably to the importance of maintaining packing plants hygienically clean in an effort to reduce this initial contamination to a minimum.

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